

## REMARKS

Claims 1-5, 7, 9, 11-20, 24, 26, 28-33, 37, 39, and 41-66 are pending. Due to a restriction requirement, claims 46-61 are withdrawn from consideration. Claims 12, 28, and 41 are objected to under 37 C.F.R. § 1.75(c) as being of improper dependent form. Claims 1-5, 7, 9, 11-20, 24, 26, 28-33, 37, 39, 41-45, and 62-66 are rejected under 35 U.S.C. § 112, first paragraph, for lack of a written description in the specification. Claims 1, 3, 5, 7, 9, 20, 24, 26, 28, 30-33, 37, 39, 41, 43-45, and 62-64 are rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. Claims 1-4 and 16-19 stand rejected under 35 U.S.C. § 103(a) as being obvious over Nagai et al. (U.S. Application Serial No. 09/728,207, now allowed), in view of Yu et al. (Genes Cells 2:457-466, 1997) and Hirsch et al. (J. Virol. 70:3741-3752, 1996). Claims 1-5, 7, 9, 16-20, 24, 26, 28-33, 37, 39, 41-45, and 62-66 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Flanagan et al. (J. Gen. Virol. 78:991-997, 1997) and Seth et al. (PNAS 95:10112, 1998) in view of Yu et al. (Genes Cells 2:457-466, 1997) and Hurwitz et al. (Vaccine 15:533-540, 1997), and as evidenced by Ourmanov et al. (J. Virol. 74:2740-2751, 2000). Claims 11-13 and 15 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Flanagan et al. (J. Gen. Virol. 78:991-997, 1997) in view of Yu et al. (Genes Cells 2:457-466, 1997), and Kast et al. (J. Immunol. 140:3186-3193, 1988). Claim 14 stands rejected under 35 U.S.C. § 103(a) as being unpatentable under Flanagan et al. (J. Gen. Virol. 78:991-997, 1997) in view of Yu et al. (Genes Cells 2:457-466, 1997), and Kast et al. (J.

Immunol. 140:3186-3193, 1988) as applied to claims 11-13 and 15, and further in view of Boutillon et al. (U.S. Patent No. 6,015,564). Claims 1-4 and 16-19 stand provisionally rejected under the judicially created doctrine of obviousness-type double patenting over claims 1, 4, 5, and 13 of co-pending Application Serial No. 09/728,207, now allowed, in view of Yu et al. (Genes Cells 2:457-466, 1997) and Hirsch et al. (J. Virol. 70:3741-3752, 1996). Applicants address each of these rejections and objections in turn below.

#### Claim amendments

Claims 1 and 3 have been canceled.

Claims 2 and 5 have been amended to delete the recitation of “part.”

In claims 2, 5, 11, 16, 17, 20, 24, and 33, the term “Sendai virus vector” has been amended to “Sendai virus gene-transfer vector.” Support for this amendment is found, for example, at page 13, lines 26-28, of the instant specification.

Claims 5, 11, 20, and 33 have been amended to recite the term “gp41.” Support for this amendment is found, for example, at page 22, lines 14-15, of the instant specification.

New claims 67-72 have been added. Support for claims 67-71 is found, for example, at page 22, lines 33-35, of the instant specification. Support for claim 72 is found, for example, at page 3, lines 25-30, page 4, line 30 to page 5, line 3, page 17, lines 1-12, page 49, lines 24-33, and in Figure 4 of the instant specification.

Applicants reserve the right to pursue any canceled subject matter in this or in a continuing application.

Objection under 37 C.F.R. § 1.75(c)

Claims 12, 28, and 41 are objected to under 37 C.F.R. § 1.75(c) as being of improper dependent form for failing to further limit the subject matter of a previous claim. In view of the amendments to claims 11, 20, and 33, the objection should be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph

*Written description*

Claims 1-5, 7, 9, 11-20, 24, 26, 28-33, 37, 39, 41-45, and 62-66 stand rejected under 35 U.S.C. § 112, first paragraph, for an asserted lack of a written description in the specification. In particular, the Office states (page 3):

[T]he central issue under this rejection is the structure-functional relationship of the HIV/SIV proteins and the parts thereof, and whether the protein and the parts thereof have the capability of serving as a vaccine for HIV/SIV. (Emphasis original.)

In support of its position, the Office cites to Matano et al. (AIDS 17:1392-1394, 2003) as stating that a full-length tat protein cannot induce or enhance a protective immune response. Applicants respectfully disagree.

Regarding Matano et al., the Office refers to the third paragraph of column 2, page

1392 and concludes that “it is apparent SeV-tat failed to influence an anti-viral response since the same outcome was shown compared to the group giving DNA-prime alone.”

Applicants submit that the Office still misconstrues what is disclosed in Matano et al. On this point Applicants note that one of the inventors of the instant invention, Dr. Tetsuro Matano, who is also one of the authors of Matano et al., explains the teachings of the Matano reference as follows.

Before the experiments demonstrated in the Matano reference were performed, several types of Tat-based AIDS vaccines were reported to show protective efficacies in macaque AIDS models (see Matano et al., page 1392, column 1, third paragraph).

Among the references cited as references 7-9 at page 1392, reference 7 (Cafaro et al., Nat. Med. 5:643-650, 1999; a copy of which is submitted herewith as Exhibit 1), discloses that induction of Tat-specific CTL response reduced setpoint plasma viral loads (SPVL). It was known that there was a statistically significant correlation between levels of vaccine-elicited CTL responses prior to challenge and the control of viremia following challenge (see, e.g., abstract of Barouch et al., J. Virol. 75:5151-5158, 2002; a copy of which is submitted herewith as Exhibit 2). Thus, induction of Tat-specific CTL response would lead to elevated levels of vaccine-induced viral-specific CTL and reduction of SPVL.

The DNA-prime/SeV-Tat-booster experiments described by Matano et al. were performed under these circumstances. As described at the second paragraph of column 2, page 1392 of Matano et al., all the animals showed the induction of SHIV (simian/human

immunodeficiency virus)-specific T cells by DNA vaccination, and after the booster, the expansion of SHIV-specific T cells, particularly SHIV-specific CD4 T cells. Thus, the DNA-prime and SeV-Tat-booster induced Tat-specific T cell responses. The animals then were challenged with SHIV. Macaques vaccinated with DNA alone and those vaccinated with DNA/SeV-Tat showed reduced levels of setpoint viral loads and were protected from progression of AIDS, except that one animal in each group showed acute CD4 T cell depletion.

Matano et al.'s results indicate that no significant differences were found in protection levels between the DNA-vaccinated and the DNA/SeV-Tat-vaccinated groups. This does not indicate that Tat cannot induce or enhance a protective immune response, but rather indicates that, after the DNA-prime alone reduced SPVL, the SeV-Tat booster failed to further reduce peak plasma viral loads. Indeed, SeV-Tat successfully induced an immune response specific to the immunodeficiency viral protein, repressed propagation of an immunodeficiency virus, and reduced SPVL. Furthermore, the Office focuses on one negative result instead of two positive results. The Matano reference does not deny effectiveness of Tat for the use recited in the present claims, as amended, and cannot be used to support the Office's position that not every HIV/SIV or a fragment thereof could serve as an AIDS vaccine.

Furthermore, the Office includes claims 11, 16, 17, 20, and 33, and their dependent claims, in the claims rejected as lacking a written description in the specification as filed.

However, Applicants note that these claims do not recite the use of a Sendai virus vector as vaccine as a claim limitation, and, therefore, should not have been included in the present written description rejection. In particular, if all the claim limitations are supported by the description of the specification, the written description requirement is met. The immunodeficiency viral proteins and parts thereof recited in claims 11, 16, 17, 20, and 33, and newly added claims 67-72 are supported by the instant description as evidenced by the following references submitted previously or at this time: Ourmanov et al., Hirsch et al., Boutillon et al., Geffen et al., Leung et al., NIAID's SPIRAT group, and Kano et al. (Gag, Pol, Env, gp41, and Gag-pol); Ensoli et al., Cafaro et al., and Matano et al. (Tat: two positive results vs. one negative result); Boutillon et al. and Ayyavoo et al. (Nef); NIAID's SPIRAT group (Rev); and Ayyavoo et al. (Vif, Vpr, and Vpu).

In view of the above, the written description rejection should be withdrawn.

#### *Enablement*

Claims 1, 3, 5, 7, 9, 20, 24, 26, 28, 30-33, 37, 39, 41, 43-45, and 62-64 stand rejected under 35 U.S.C. § 112, first paragraph, for an asserted lack of enablement. To expedite prosecution, Applicants have canceled claims 1 and 3. The rejection of these claims is moot.

In addition, claims 2, 4, 5, 7, 9, and 62 have been amended to limit the immunodeficiency viral proteins to Gag, Pol, gp41, and Gag-Pol fusion protein. The

Office states (page 5):

[T]he specification supplemented by the state of the art, while being enabling for intranasally administering a sendai virus vector expressing a protein of an immunodeficiency virus selected from the group consisting of Gag, Pol, gp41, Gag-pol, does not reasonably provide enablement for obtaining a vaccine effect by intranasally administering a sendai viral vector expressing parts of Gag, Pol, gp41, Gag-pol proteins, or expressing the tat, rev, vap, vpx vpr vif nef proteins, and parts thereof.

As claims 2, 4, 5, 7, 9, and 62 have been amended to be directed to subject matter that the Office deems to be enabled by the specification and the state of the art, Applicants submit that the enablement rejection of these claims, as amended, may be withdrawn.

The other claims included in this basis for rejection, and the newly added claims, do not recite the use of a Sendai virus vector as a vaccine. As discussed above and as evidenced by the references listed above, these claims are clearly enabled by the present specification.

Furthermore, the Office asserts that Applicants fail to address the concerns regarding genomic DNA of immunodeficiency virus as indicated at page 15 of the Office Action mailed on January 26, 2005. However, Applicants submit that the Office's concerns are addressed at the paragraph bridging pages 20 and 21 of the previous response.

For all the above reasons, the rejection for lack of enablement of the present claims should be withdrawn.

### Rejections under 35 U.S.C. § 103(a)

Claims 1-4 and 16-19 stand rejected under 35 U.S.C. § 103(a) as being obvious over Nagai et al. (U.S. Application Serial No. 09/728,207, now allowed), in view of Yu et al. (Genes Cells 2:457-466, 1997) and Hirsch et al. (J. Virol. 70:3741-3752, 1996). As submitted in the previous response, Applicants will address this rejection, if appropriate, upon an indication of allowable subject matter by filing a statement indicating that U.S. Application Serial No. 09/728,207 and the present application were, at the time the present application was filed, commonly owned.

Claims 1-5, 7, 9, 16-20, 24, 26, 28-33, 37, 39, 41-45, and 62-66 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Flanagan et al. (J. Gen. Virol. 78:991-997, 1997) and Seth et al. (PNAS 95:10112, 1998) in view of Yu et al. (Genes Cells 2:457-466, 1997) and Hurwitz et al. (Vaccine 15:533-540, 1997), and as evidenced by Ourmanov et al. (J. Virol. 74:2740-2751, 2000). Claims 11-13 and 15 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Flanagan et al. (J. Gen. Virol. 78:991-997, 1997) in view of Yu et al. (Genes Cells 2:457-466, 1997), and Kast et al. (J. Immunol. 140:3186-3193, 1988). Claim 14 stands rejected under 35 U.S.C. § 103(a) as being unpatentable under Flanagan et al. (J. Gen. Virol. 78:991-997, 1997) in view of Yu et al. (Genes Cells 2:457-466, 1997), and Kast et al. (J. Immunol. 140:3186-3193, 1988) as applied to claims 11-13 and 15, and further in view of Boutillon et al. (U.S. Patent No. 6,015,564). To expedite prosecution, Applicants have amended the claims to replace



“Sendai virus vector” with “Sendai virus gene-transfer vector.” In view of this amendment, as noted below, Applicants submit that the obviousness rejections should be withdrawn.

At page 21 of the Office Action mailed on January 26, 2005, the Office states that Yu et al. teach that “the V(-) version appears to be excellent and almost comparable to the above noted VV-based expression” (column 1, page 462) and concludes that Yu et al. teach that Sendai virus could be used as carrier for expressing a analogous viral protein, such as the immunodeficiency virus, in place of the vaccinia virus or interchangeably with other known viral vectors. It should be noted that, just after the sentence quoted by the Office, Yu et al. teach that “SeV-based expression is a novel, useful option for producing large quantities of gp120 from mammalian cells.” Furthermore, at Conclusions of Abstract, Yu et al. disclose “SeV-based expression serves as a novel choice for producing large quantities of HIV-1 gp120 and will greatly facilitate biochemical, biological and immunological studies of this important glycoprotein.” It is clear from these teachings that Yu et al. disclose the use of Sendai virus as *an expression vector*. The sentence quoted by the Office merely compares Sendai virus with vaccinia virus as an expression vector. Yu et al. do not teach or suggest that Sendai virus can be used as a gene-transfer vector.

In the absence of a teaching or suggestion that Sendai virus can be used as a gene-transfer vector, Applicants submit that one skilled in the art would not be motivated to

replace adenovirus taught in Flanagan et al. or vaccinia virus taught in Seth et al. with Sendai virus.

The Office also asserts that Hurwitz et al. teach the advantage of using Sendai virus as a potential human vaccine (see Office Action mailed January 26, 2005, bottom of page 21). Indeed, Hurwitz et al. teach that Sendai virus itself can be used as an antigen. Nonetheless, Hurwitz et al. neither suggest nor disclose the use of Sendai virus as a gene-transfer vector encoding and expressing a heterologous viral protein.

Similar to Hurwitz et al., Kast et al. teach that Sendai virus itself can be used as an antigen, but fail to teach its use as a gene-transfer vector. Nothing is disclosed about Sendai virus vectors in Flanagan, Seth, Ourmanov, or Boutillon.

To establish a *prima facie* case of obviousness, the prior art references must teach or suggest all the claim limitations. As discussed above, none of the prior art references suggest or disclose the use of a Sendai virus vector as a gene-transfer vector.

Furthermore, in response to Applicants' argument that Yu et al. fail to yield functional luciferase, the Office states that the problem in assessment appears not in the failure to yield functional luciferase, but over-production leads to "extensive aggregation of the expressed luciferase molecules in cells" (page 10, first paragraph of the present Office Action). This counterargument is not on point. The sentences quoted by the Office merely describe that a foreign gene can be readily inserted into the Sendai viral cDNA. Although Yu et al. describe the foreign gene insertion site in Sendai virus, a

luciferase gene inserted in the insertion site was overexpressed and the expression product was aggregated so as not to be functional. It is not important here to discuss the reasons for failure to obtain functional luciferase; what is important is the fact that functional luciferase could not be obtained regardless of the reasons. From the teachings of Yu et al., it is questionable whether a Sendai virus vector encoding a foreign gene could produce a foreign gene product that is functional to induce an immune response specific to the product. If the product aggregates, an immune response specific to the intact form of the product may not be induced. Therefore, even if the teachings of Yu et al. are combined with those of the other prior art references, one skilled in the art would not have reasonable expectation of success for inducing an immune response specific to an antigen that is encoded and expressed by a Sendai virus vector.

In view of the above, the rejections for obviousness over the cited art should be withdrawn.

Rejection under the judicially created doctrine of obviousness-type double patenting

Claims 1-4 and 16-19 stand provisionally rejected for obviousness-type double patenting over claims 1, 4, 5, and 13 of copending Application Serial No. 09/728,207, now allowed, in view of Yu et al. (Genes Cells 2:457-466, 1997) and Hirsch et al. (J. Virol. 70:3741-3752, 1996). Applicants respectfully request that the provisional rejection be held in abeyance until such time as allowable subject matter is identified.

## CONCLUSION

Applicants submit that the claims are now in condition for allowance, and this action is hereby respectfully requested.

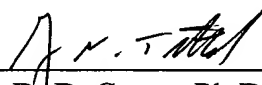
Enclosed is a Petition to extend the period for replying to the final Office Action for three months, to and including April 24, 2006, and a check in payment of the required extension fee. Also enclosed is a Notice of Appeal in which Applicants appeal the final rejection of claims 1-5, 7, 9, 11-20, 24, 26, 28-33, 37, 39, 41-45, and 62-66.

Further, enclosed is a check in the amount of \$200.00 in payment of excess claims fees for four (4) excess dependent claims added.

If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 24 April 2006

  
\_\_\_\_\_  
James D. DeCamp, Ph.D.  
Reg. No. 43,580

Clark & Elbing LLP  
101 Federal Street  
Boston, MA 02110  
Telephone: 617-428-0200  
Facsimile: 617-428-7045

JAMES D. DE CAMP, Ph.D.  
Reg. No. 52,290

# Control of SHIV-89.6P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine

AURELIO CAFARO<sup>1</sup>, ANTONELLA CAPUTO<sup>2</sup>, CLAUDIO FRACASSO<sup>1</sup>, MARIA T. MAGGIORELLA<sup>1</sup>,  
DELIA GOLETTI<sup>1</sup>, SILVIA BARONCELLI<sup>1</sup>, MONICA PACE<sup>1</sup>, LEONARDO SERNICOLA<sup>1</sup>,  
MARTIN L. KOANGA-MOGTOMO<sup>1</sup>, MONICA BETTI<sup>2</sup>, ALESSANDRA BORSETTI<sup>1</sup>, ROBERTO BELLI<sup>1</sup>,  
LENNART ÅKERBLÖM<sup>3</sup>, FRANCO CORRIAS<sup>1</sup>, STEFANO BUTTÒ<sup>1</sup>, JONATHAN HEENEY<sup>4</sup>,  
PAOLA VERANI<sup>1</sup>, FAUSTO TITTI<sup>1</sup> & BARBARA ENSOLI<sup>1</sup>

<sup>1</sup>Laboratory of Virology, Istituto Superiore di Sanità, 00161 Rome, Italy; <sup>2</sup>Department of Experimental and Diagnostic Medicine, University of Ferrara, 44100 Ferrara, Italy; <sup>3</sup>Department of Virology, The National Veterinary Institute, Biomedical Centre, S-751 83 Uppsala, Sweden; <sup>4</sup>Department of Virology, Biomedical Primate Research Centre, Rijswijk ZH 2288 GJ, The Netherlands  
Correspondence should be addressed to B.E.; email: ensoli@virus1.net.iss.it

Vaccine strategies aimed at blocking virus entry have so far failed to induce protection against heterologous viruses. Thus, the control of viral infection and the block of disease onset may represent a more achievable goal of human immunodeficiency virus (HIV) vaccine strategies. Here we show that vaccination of cynomolgus monkeys with a biologically active HIV-1 Tat protein is safe, elicits a broad (humoral and cellular) specific immune response and reduces infection with the highly pathogenic simian-human immunodeficiency virus (SHIV)-89.6P to undetectable levels, preventing the CD4<sup>+</sup> T-cell decrease. These results may provide new opportunities for the development of a vaccine against AIDS.

Most AIDS vaccine strategies (reviewed in refs. 1, 2) have failed to protect against heterologous viruses because of the HIV-1 Envelope strain variability<sup>3-5</sup>. Vaccines with live attenuated viruses can protect against heterologous viruses<sup>1,6-8</sup>; however, delayed disease onset and the appearance of revertant viruses<sup>9-11</sup> hamper their use in humans.

We chose to target the Tat protein of HIV because Tat is produced early after infection and is essential for virus replication and infectivity<sup>12-14</sup>. In addition, Tat is released extracellularly by infected cells<sup>15-18</sup> and is taken up by neighbor cells where activates virus replication<sup>16,19,20</sup>. Extracellular Tat also favors transmission of both macrophage-tropic and T cell-tropic HIV-1 strains by inducing CCR5 and CXCR4 co-receptors<sup>21,22</sup>. Tat is also essential in the pathogenesis of AIDS pathogenesis and AIDS-associated Kaposi's sarcoma<sup>14,15,18,20,23-30</sup>.

Tat is also immunogenic, and antibodies against Tat may have protective effects in controlling disease progression<sup>31-34</sup> by inhibiting both the effect of extracellular Tat on HIV replication<sup>16,35</sup> and its immunosuppressive effects on T cells<sup>36</sup>. Furthermore, the presence of anti-Tat cytotoxic T lymphocytes (CTLs) in the initial phase of infection correlates inversely with progression to AIDS (refs. 35-37). Tat protein is efficiently taken up by cells<sup>16,17,19,38</sup> and can induce CD8<sup>+</sup> T cell-mediated CTL-responses by entering the major histocompatibility complex (MHC) class I pathway<sup>39</sup>. Finally, Tat is conserved in its immunogenic epitopes among the different subtypes, with the exception of the O subtype<sup>40</sup>.

Thus, although a Tat vaccine cannot block virus entry, the immune response to Tat may control virus replication and transmission. As a result, the infection could be confined and progression to AIDS could be blocked, as has been suggested<sup>41</sup>.

## Vaccination of cynomolgus monkeys with Tat

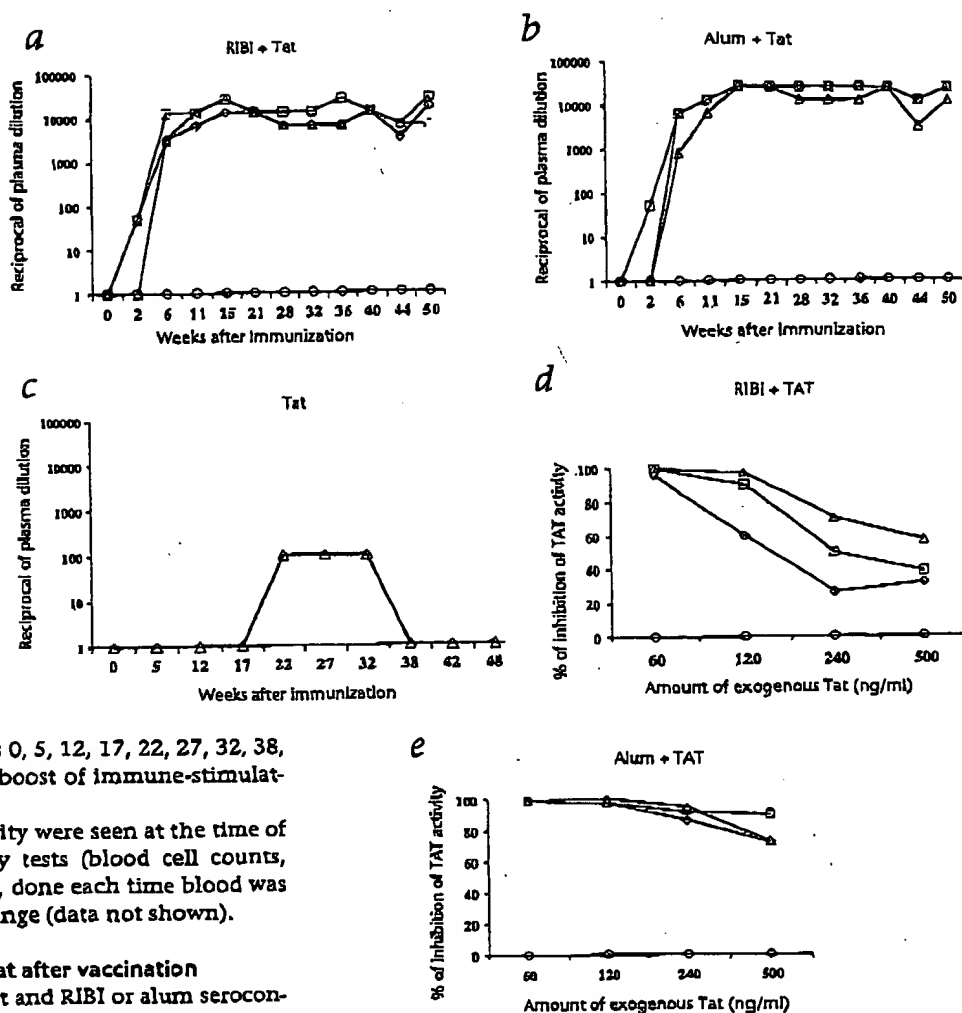
We immunized seven cynomolgus monkeys (*Macaca fascicularis*) with a biologically active Tat protein<sup>17</sup> (Table 1). Six monkeys were immunized subcutaneously with Tat (10 µg) and the adjuvant RIBI (RIBI; *n* = 3) or the adjuvant aluminum phosphate (alum; *n* = 3), and one monkey, with Tat (6 µg), intradermally, in the absence of adjuvants. Two control monkeys were injected subcutaneously with either RIBI or alum alone. A naive monkey was included in the protocol at the time of challenge as an additional control. Boosts were given at 2, 6, 11, 15, 21, 28, 32 and 36 weeks after the first immunization. The last boost was given intramuscularly with Tat in immune-stimulating complexes<sup>42</sup>. Monkey 54222, immunized intradermally, was vaccinated on a

Table 1 Vaccination protocols

Monkey	Immunogen	Adjuvant	Administration
54844	Tat protein	RIBI (250 µl)	SC, 500 µl in one site
54879	(10 µg/250 µl)		(dorsal area, near the neck)
54963	Tat protein	alum (250 µl)	SC, 500 µl in one site
54899	(10 µg/250 µl)		(dorsal area, near the neck)
55396	Tat protein		ID, 150 µl in two sites
54240	(6 µg/300 µl)		(upper dorsal area)
54222	buffer	RIBI (250 µl)	SC, 500 µl in one site
55123	(250 µl)		(dorsal area, near the neck)
55129	buffer	alum (250 µl)	SC, 500 µl in one site
	(250 µl)		(dorsal area, near the neck)
12	None	None	

Six macaques were immunized subcutaneously (SC) with Tat in saline containing 20% autologous serum and RIBI or alum. One monkey was vaccinated intradermally (ID) with Tat. Control monkeys received RIBI or alum alone. Monkey 12, control of the virus inoculum at the time of challenge.

Fig. 1 Humoral responses to Tat. **a-c**, Titers of antibody against Tat after the first immunization (week 0). Monkeys were inoculated with: **a**, RIBI + Tat: 54844 (○), 54879 (□), 54963 (Δ) or RIBI alone: 55123 (○); **b**, alum + Tat: 54899 (○), 55396 (□), 54240 (Δ) or alum alone: 55129 (○); or **c**, Tat alone: 54222 (Δ). Titers represent the reciprocal of the last plasma dilution at which the test was still positive. **d-e**, Percentages of inhibition by plasma (1:2 dilution) from vaccinated monkeys of the rescue of Tat-defective proviruses induced by 60–500 ng/ml Tat. Plasma obtained at week 21 after immunization. Macaques were vaccinated with: **d**, RIBI + Tat: 54844 (○), 54879 (□), 54963 (Δ); or **e**, alum + Tat: 54899 (○), 55396 (□), 54240 (Δ). Controls (○), pooled pre-immune plasma from the corresponding groups.



slightly different schedule (on weeks 0, 5, 12, 17, 22, 27, 32, 38, 42 and 48) and did not receive the boost of immune-stimulating complexes.

No signs of local or systemic toxicity were seen at the time of vaccination, and clinical laboratory tests (blood cell counts, blood chemistry and FACS analysis), done each time blood was drawn, were always in the normal range (data not shown).

#### Humoral and cellular responses to Tat after vaccination

The six monkeys inoculated with Tat and RIBI or alum seroconverted by week 6 after the first immunization, and the antibody titers increased up to 1:25,600 in all monkeys immunized with Tat and alum, and up to 1:12,800 in the monkeys immunized with Tat and RIBI; these titers remained stable up to 50 weeks after the first immunization (Fig. 1a and b). Monkey 54222 (given Tat intradermally) developed low titers of antibodies against Tat (1:100), which were detected up to 32 weeks after immunization (Fig. 1c).

In addition, at week 21 after immunization, plasma from the six macaques inoculated with Tat and RIBI or alum were capable of neutralizing the activity of 120–500 ng/ml of Tat on HIV-1 replication, compared with pre-immune plasma (Fig. 1d–e), as shown by the inhibition of the rescue of Tat-defective proviruses induced after the addition to the cells of serial concentrations of Tat (refs. 16,17,20).

Furthermore, at week 44 after immunization, plasma from monkeys 54963 (given RIBI and Tat) and 54899 and 55396 (given alum and Tat) were capable of neutralizing the replication of the SHIV89.6P after *in vitro* acute infection of CEMx174 cells, compared with pre-immune plasma (data not shown). In both assays, neutralization correlated with the titers of antibody against Tat.

Tat-specific proliferation was seen in three of three monkeys vaccinated with Tat and RIBI (Fig. 2a–c) and in three of three vaccinated with Tat and alum (Fig. 2e–g), whereas no response was detected in the macaque

Table 2 Tat-specific CTLs and TNF- $\alpha$  production in vaccinated monkeys

Group	Monkey	Specific killing (%) <sup>a</sup>				TNF- $\alpha$ (pg/ml) <sup>b</sup>		
		Week 28		Week 36		None	PHA	Tat
RIBI + Tat (10 $\mu$ g, SC)	54844	50:1	25:1	50:1	25:1	0	958	70
	54879	ND	ND	ND	ND	0	110	260
	54963	0	0	0	0	ND	ND	ND
ALUM + Tat (10 $\mu$ g, SC)	54899	4.7	3.9	15	8.3	0	9	344
	55396	1.1	1.1	0	1	0	416	0
	54240	7.2	2.6	ND	2.6	0	0	150
Tat (6 $\mu$ g, ID)	54222 <sup>c</sup>	1.2	0	ND	12.4 <sup>d</sup>	0	30	50
Control (RIBI)	55123	ND	ND	0	0	0	40	0
Control (alum)	55129	ND	ND	0	0	0	60	0

<sup>a</sup>Anti-Tat CTL activity of PBMCs; Anti-Tat CTL activity of PBMCs at 50:1 and 25:1 effector:target ratios, weeks 28 and 36, after immunization. Values greater than 10% were considered positive. Monkey 54879 could not be tested because B-cell transformation with Papio herpesvirus failed despite several attempts. <sup>b</sup>TNF- $\alpha$  production from PBMCs obtained at week 44 after immunization; values represent the average of duplicate wells. Values below cut-off (15.6 pg/ml) were given a value of 0. <sup>c</sup>Weeks 22 and 32 after immunization; <sup>d</sup>12.5:1 effector:target ratio. ND, not done; SC, subcutaneously; ID, intradermally.

vaccinated intradermally with Tat alone (data not shown) and in the control monkeys (Fig. 2d and 2h).

Specific anti-Tat CTL activity began to be detectable in the vaccinated monkeys at week 28 after immunization, but only reached levels above the cut-off (10%) at week 36 in one of two macaques vaccinated with Tat and RIBI, in one of three monkeys vaccinated with Tat and alum and in the monkey vaccinated with Tat alone (intradermally) (Table 2). Although at week 28 monkey 54240 (given alum and Tat) showed detectable but low (below 10%) CTL activity, at week 36 the test was inconclusive.

To confirm and extend these data, at week 44 after immunization, we tested the production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a known mediator of CTL activity<sup>43-46</sup>, after stimulation of peripheral blood mononuclear cells (PBMCs) with Tat or phytohemagglutinin (PHA). Monkeys 54879 and 54240 (for which CTL activity could not be tested or was inconclusive, respectively) and the monkeys that showed anti-Tat CTL activity all produced

**Table 3** TNF- $\alpha$  production by CD8<sup>+</sup> and CD8<sup>-</sup> cells after Tat stimulation, and correlation with cell phenotype

Group	Monkey	TNF- $\alpha$ (pg/ml)		CD8 <sup>+</sup> cells (%)	CD3 <sup>+</sup> /CD8 <sup>+</sup>	CD3 <sup>+</sup> /CD8 <sup>-</sup>
		CD8 <sup>+</sup> cells	CD8 <sup>-</sup> cells	Total		
RIBI + Tat (10 $\mu$ g, SC) alum + Tat (10 $\mu$ g, SC)	54879	2	22	88.6	93.4	6.6
	54899	2	19	72	85	15
	54240	1	7	74.2	94.5	5.5

Week 44 after immunization. Percentage of total CD8<sup>+</sup> cells, CD3<sup>+</sup>/CD8<sup>+</sup> T cells and CD3<sup>+</sup>/CD8<sup>-</sup> NK cells. SC, subcutaneously.

TNF- $\alpha$  after Tat stimulation (Table 2). In contrast, monkeys lacking CTL activity, including the control monkeys, produced TNF- $\alpha$  after stimulation with PHA but not with Tat (Table 2).

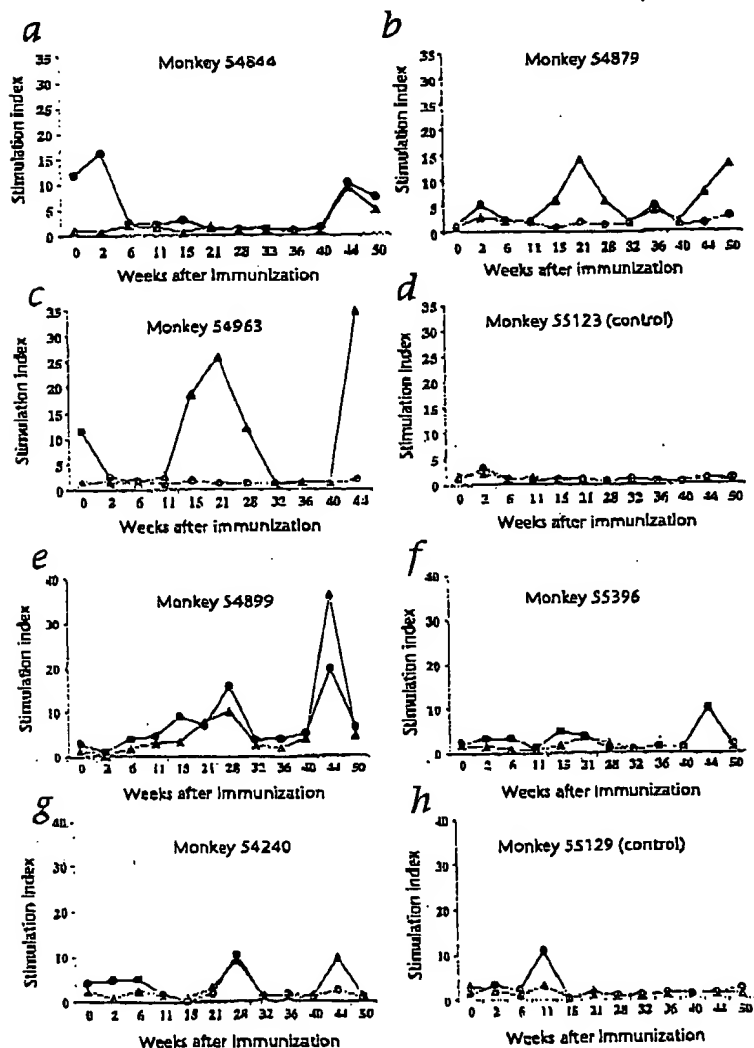
To determine the cell source of TNF- $\alpha$  after Tat stimulation, we separated PBMCs from three of the five responsive monkeys (54879, 54899 and 54240, for which cells were available) into CD8<sup>+</sup> and CD8<sup>-</sup> subsets and evaluated Tat-induced TNF- $\alpha$  production separately in the two cell fractions, which we also analyzed by FACS. The main source of TNF- $\alpha$  (~90%) was the CD8<sup>+</sup> cell fraction, which was mostly (range, 85–94.5%) T cells (CD3<sup>+</sup>/CD8<sup>+</sup>) with a minority (range, 5.5–15%) of NK cells (CD3<sup>+</sup>/CD8<sup>-</sup>) (Table 3).

From week 11 or 15 after primary immunization, skin tests to Tat produced positive results in two of three monkeys inoculated with Tat and RIBI (54879 and 54963) and in three of three monkeys inoculated with Tat and alum (data not shown). Monkey 54222 (given Tat intradermally) did not show any reactivity to Tat. All monkeys responded to the recall antigen tetanus toxoid.

#### Challenge with SHIV89.6P

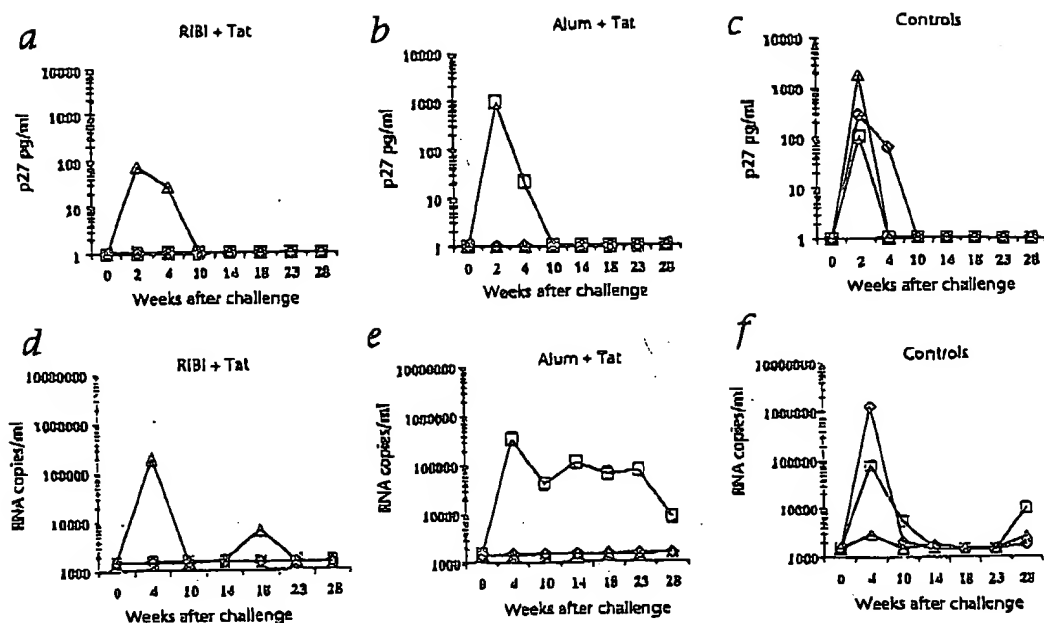
We used SHIV-89.6P for the virus challenge because it is highly pathogenic in macaques and because it contains the *tat* gene of HIV-1 (ref. 47). The virus stock used for challenge was derived from a cynomolgus macaque inoculated with the original SHIV89.6P from rhesus monkeys.

To determine virus pathogenicity in cynomolgus and the monkey infectious dose (MID<sub>50</sub>), we inoculated virus stocks obtained from rhesus and cynomolgus macaques into six and eight monkeys, respectively. There were high levels of virus replication and a profound and persistent decrease in CD4 T cells in all monkeys inoculated with each virus stock (from 2,852 to 2.8 MID<sub>50</sub>) (data not shown), as described<sup>47</sup>. Therefore, we challenged all vaccinated macaques and the two control monkeys intravenously with 10 MID<sub>50</sub> of SHIV-89.6P. As an additional control, we inoculated a naïve monkey (12) with 2.8 MID<sub>50</sub> of the virus, a dose lower than the challenge dose.



**Fig. 2** Lymphoproliferative responses.  $\Delta$ , Tat;  $\bullet$ , tetanus toxoid. Monkeys were inoculated with: a–c, RIBI + Tat; d, RIBI alone (control); e–g, alum + Tat; or h, alum alone (control). Vertical axis, fold of proliferation (measured by <sup>3</sup>H-thymidine incorporation) of antigen-stimulated cells compared with unstimulated cells (stimulation index); values greater than 3 (cut-off) were considered positive. Monkey 54222, inoculated with Tat alone, responded to tetanus toxoid (fivefold to tenfold) but not to Tat (data not shown). Response to PHA, positive control (stimulation index  $\geq$  4).

Fig. 3 Detection of viral infection up to 28 weeks after challenge with SHIV-89.6P. **a–c**, Detection of p27 'antigenemia'. **d–f**, Detection of plasma viremia (SHIV RNA copies/ml plasma). Monkeys were inoculated with: **a** and **d**, RIBI + Tat: 54844 (◇), 54879 (□), 54963 (Δ); **b** and **e**, alum + Tat: 54899 (◇), 55396 (□), 54240 (Δ); or **c** and **f**, RIBI alone: 55123 (◇), alum alone: 55129 (□) or nothing: 12 (Δ).



After challenge, all three control monkeys were infected, as indicated by the presence of p27 antigen (Fig. 3a–c) and viral RNA (Fig. 3d–f) in plasma. In contrast, only two vaccinated monkeys (54963, given Tat and RIBI, and 55396, given Tat and alum) were infected, as shown by both assays (Fig. 3). For the other five vaccinees, including monkey 54222 (given Tat alone), p27 and viral RNA were always undetectable. Moreover, PBMCs from both the control macaques and the two vaccinated, infected monkeys had a high proviral copy number from week 2 after challenge (Fig. 4a–c). One other macaque (54879, given Tat and RIBI), had a very low proviral copy number (1.5 copies/μg DNA) only at week 14 after challenge and not later (Fig. 4a–c). In contrast, in all the other vaccinated monkeys, including monkey 54222 (given Tat alone), proviral DNA was always undetectable.

The control monkeys and the two vaccinated and infected monkeys (54963 and 55396) also had consistently high levels of cell associated viral load (cytoviremia) (Fig. 4d–f). In contrast, the other five vaccinated macaques were always negative, including monkey 54222 (given Tat alone). Moreover, each attempt to isolate virus (done since week 14 after challenge) from PBMCs depleted of CD8<sup>+</sup> T cells and stimulated with PHA from these five monkeys failed (Fig. 4).

To verify that infection had occurred also in the 'protected' monkeys, we did serological assessments. Antibodies against SIV were detected in all the macaques from week 2 or 4 after challenge (Fig. 5). Control monkeys had the highest titers (at least 2 logs higher) and these increased rapidly compared with those of the vaccinated monkeys. The two infected, vaccinated monkeys, however, had higher titers than the five 'protected' monkeys, but these were delayed compared with those of the control monkeys. Moreover, in the five 'protected' monkeys, titers of antibody against SIV became undetectable from week 14 or 18 after challenge. Antibodies against the HIV-1 Env of SHIV89.6P were detected in plasma from three of three control monkeys and in the two vaccinated and infected monkeys, but not in the other monkeys (data not shown). However, they were detected by *in vitro* antibody production in the super-

natants of cultured PBMCs stimulated with mitogen pokeweed (PWM) from all the monkeys, with the highest titers in the control monkeys (data not shown). Thus, infection probably had occurred in all monkeys after challenge; however, in five of seven vaccinated monkeys, it was kept at a very low or undetectable level.

Consistent with these data, the CD4<sup>+</sup> T-cell counts decreased considerably in both the control monkeys and the two vaccinated and infected monkeys, whereas they remained high and stable in the five 'protected' macaques (Fig. 6). An increase in CD8<sup>+</sup> cells was detected in all monkeys after challenge. However, at week 23 or 28 after challenge, the number of CD8<sup>+</sup> lymphocytes returned to values similar to values before challenge (Fig. 6).

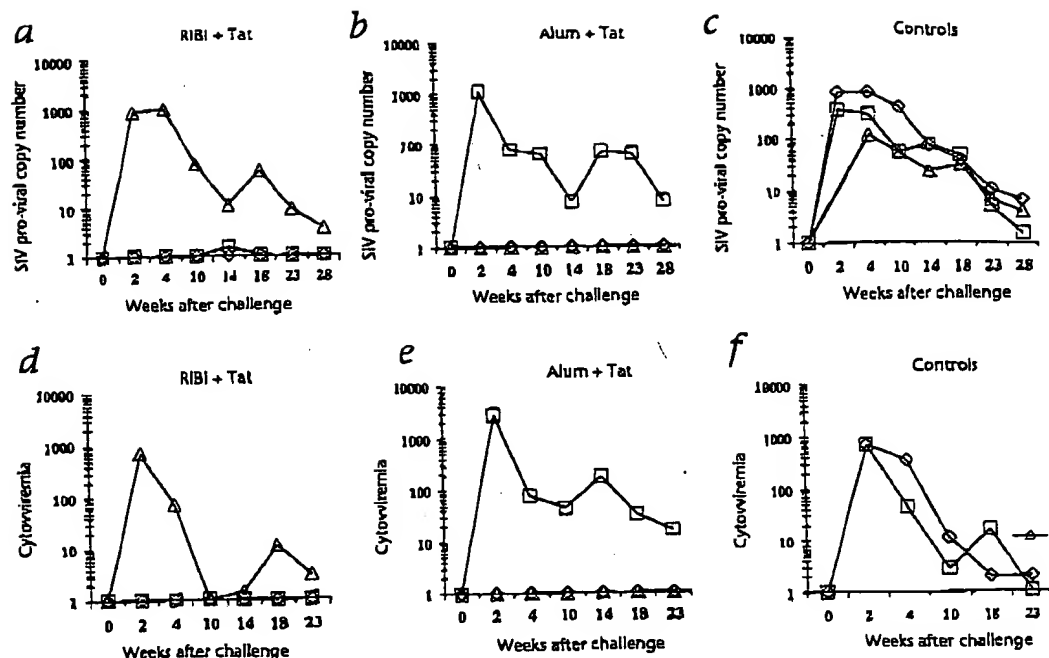
#### Discussion

These data indicate that immunization with a biologically active Tat protein is safe and induces a complete Tat-specific immune response. Vaccination with Tat controlled (up to 28 weeks after challenge) infection with the highly pathogenic virus SHIV-89.6P in five of seven vaccinated monkeys. In these monkeys, the CD4<sup>+</sup> T-cell numbers did not decrease after challenge, confirming that the Tat vaccine blocked virus replication and, consequently, prevented the CD4<sup>+</sup> T-cell decrease. All parameters of virus replication and a profound CD4 T-cell decrease were seen in all the naive monkeys ( $n = 16$ , including the controls of the vaccination protocol) inoculated in our lab with different MID<sub>50</sub> (from 2,852 to 2,852) of SHIV89.6P (grown in Rhesus or in cynomolgus), except in five of seven of the Tat-vaccinated monkeys.

Although high titers of neutralizing antibodies against Tat were present in all vaccinated monkeys (except for monkey 54222, inoculated with Tat intradermally), two of three of the monkeys capable of neutralizing both Tat activity and *in vitro* SHIV replication were infected after challenge. No virus was detected in the monkey inoculated with Tat alone, which had low antibody titers. In contrast, anti-Tat CTLs and/or Tat-induced TNF-α production were found in all 'protected' monkeys, but



**Fig 4** Detection of viral infection after challenge with SHIV-89.6P. **a-c**, Quantitation of the SIV proviral copy number (copies/ $\mu$ g DNA) up to 28 weeks after challenge. **d-f**, Cyto-viremia (infected cells/ $10^6$  PBMCs) or virus isolation up to 23 weeks after challenge. From week 14 after challenge, all monkeys negative in cyto-viremia were tested for virus isolation (instead of cyto-viremia) after CD8<sup>+</sup> T-cell depletion and stimulation with PHA and rIL-2, and the results were always negative. Monkeys were inoculated with: **a** and **d**, RIBI + Tat: 54844 ( $\diamond$ ), 54879 ( $\square$ ), 54963 ( $\Delta$ ); **b** and **e**, alum + Tat: 54899 ( $\diamond$ ), 55396 ( $\square$ ), 54240 ( $\Delta$ ); or **c** and **f**, RIBI alone: 55123 ( $\diamond$ ), alum alone: 55129 ( $\square$ ) or nothing: 12 ( $\Delta$ ). **c**, For monkey 12 (control monkey inoculated with 2.8 MID<sub>50</sub> of SHIV), DNA PCR was also positive at week 2, however, the proviral copy number was not determined. **f**, Before week 23, mon-



key 12 was always tested by virus isolation and results were always positive (data not shown).

they were undetectable in the two infected, vaccinated monkeys. Most of this TNF- $\alpha$  originated from the CD8<sup>+</sup> T-cell subset, confirming that CTLs detected in the monkeys belong to this cell type<sup>39</sup>.

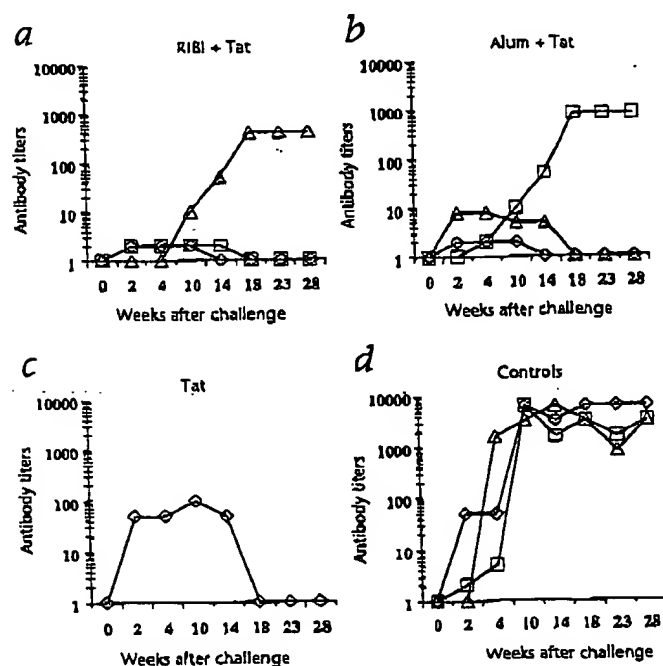
The low levels of the anti-Tat CTLs detected before challenge in our monkeys are not surprising, because although Tat-specific CTLs responses can interfere with disease progression<sup>36</sup>, the CTL levels are generally much lower and therefore are not com-

parable with those detected against other viral proteins such as Gag, Pol or Env (refs. 36,37,48-50).

These data indicate that a cellular response to Tat may be involved in controlling infection, as suggested by previous work<sup>36</sup> and by data showing an inverse correlation between the presence of CTLs and HIV-1 viral load<sup>40</sup>. However, these data do not exclude the possibility of a role for antibodies against Tat in controlling infection and progression to AIDS. In particular, the two vaccinated, infected monkeys had slowly increasing and lower titers of antibodies against SIV or HIV, compared with those of the control monkeys, indicating that neutralizing antibodies against Tat may interfere with virus replication.

An AIDS vaccine based on Tat represents a strategy aimed at controlling HIV replication and at modifying the virus-host interaction that leads to progressive immunodeficiency and disease onset. However, the immune response against Tat cannot block virus entry. In fact, it is likely that a low level and/or abortive infection has occurred also in the five 'protected' monkeys, as indicated by the presence of transient and low titers of antibody against SIV.

Sera from HIV-1-infected Ugandan patients, infected with the A and D subtypes of HIV-1 (ref. 51), recognize our Tat protein (derived from an HIV-1, subtype B strain) (S.B. *et al.*, unpublished data), which indicates that Tat is sufficiently conserved to be an optimal vaccine target against infection with all or



**Fig. 5** Titers of antibody against HIV-2/SIV up to 28 weeks after challenge with SHIV-89.6P. Monkeys were inoculated with: **a**, RIBI + Tat: 54844 ( $\diamond$ ), 54879 ( $\square$ ), 54963 ( $\Delta$ ); **b**, alum + Tat: 54899 ( $\diamond$ ), 55396 ( $\square$ ), 54240 ( $\Delta$ ); **c**, Tat alone: 54222 ( $\diamond$ ); or **d**, RIBI alone: 55123 ( $\diamond$ ), alum alone: 55129 ( $\square$ ) or nothing: 12 ( $\Delta$ ). Titers represent the reciprocal of the last dilution at which plasma were still positive.

**Fig. 6** CD4<sup>+</sup> and CD8<sup>+</sup> T-cell counts up to 28 weeks after challenge with SHIV-89.6P. **a, c, e and g.** Numbers of CD4<sup>+</sup> T cells/ $\mu$ l; **b, d, f and h.** Numbers of CD8<sup>+</sup> T cells/ $\mu$ l. Monkeys were inoculated with: **a and b,** RIBI + Tat: 54844 ( $\diamond$ ); 54879 ( $\square$ ), 54963 ( $\Delta$ ); **c and d,** alum + Tat: 54899 ( $\diamond$ ), 55396 ( $\square$ ), 54240 ( $\Delta$ ); **e and f,** Tat alone: 54222 ( $\diamond$ ); or **g and h,** RIBI alone: 55123 ( $\diamond$ ), alum alone: 55129 ( $\square$ ) or nothing: 12 ( $\Delta$ ).

most viral strains. Because Tat delivered as DNA or as a Tat toxoid is safe and immunogenic in animals and humans<sup>14,18,22-24</sup>, Tat, alone or combined with other viral products, may represent an optimal target for AIDS vaccine development for both preventive and therapeutic applications.

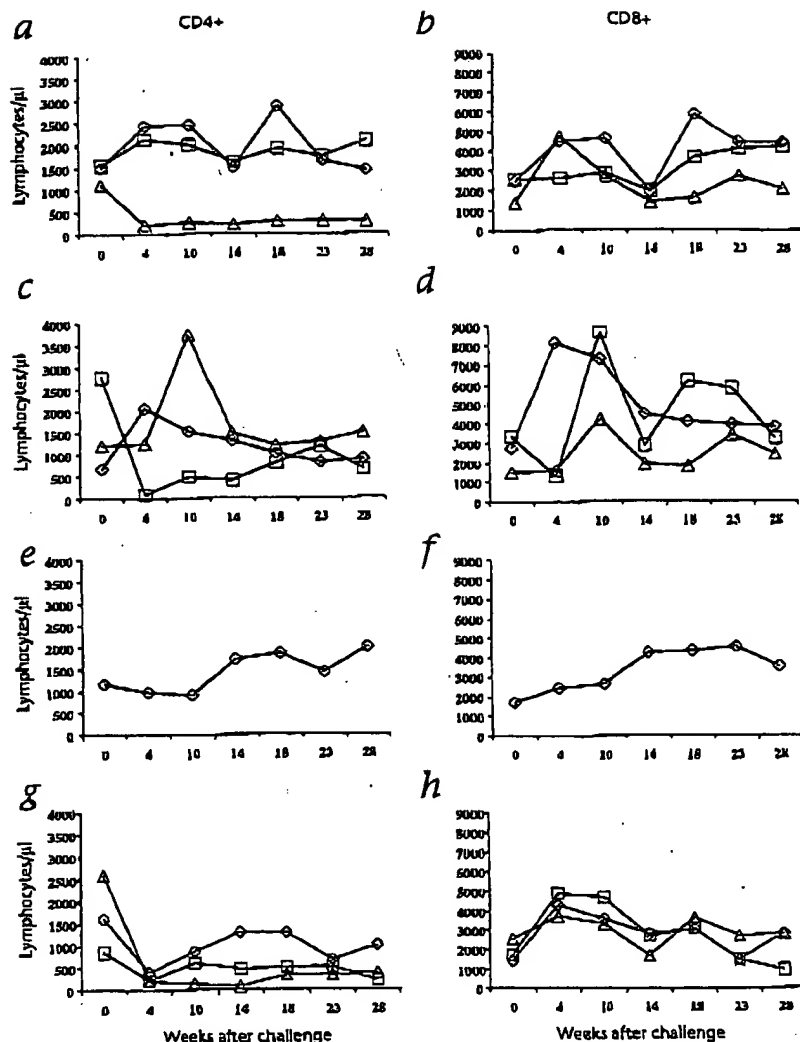
## Methods

**Animals.** Adult cynomolgus monkeys (*Macaca fascicularis*) were housed in single cages within level 3 biosafety facilities according to the European guidelines for non-human primate care (EEC, Directive No. 86-609, Nov. 24, 1986). Monkeys were examined and their weights and rectal temperatures were measured while they were sedated by ketamine hydrochloride anaesthesia (10 mg/kg). Blood samples for hematological, immunological and virological analysis were obtained in the morning before food administration.

**HIV-1 Tat protein expression, purification and inoculation.** HIV-1 Tat from the HTLV-IIIb isolate (subtype B) was expressed in *Escherichia coli*, purified to homogeneity by heparin-affinity chromatography and high-performance liquid chromatography and stored lyophilized at -80 °C as described<sup>17</sup>. Purified Tat had full biological activity in several assays<sup>15-17</sup>. As Tat is sticky, oxidizes easily and is photo- and thermo-sensitive<sup>16</sup>, it was resuspended in degassed buffer before use *in vitro*<sup>17</sup> or in saline containing 20% of autologous serum for monkey injection. Plasticware, syringes and needles were rinsed in medium containing 10% fetal calf serum (FCS) or in saline containing 20% autologous serum, and all of these procedures were done in the dark with samples on ice.

**Adjuvants.** RIBI containing monophosphoryl lipid A, trehalose dimycolate, cell wall skeleton in oil (squalene) and Tween 80 was obtained through the European Concerted Action on Macaque models for AIDS Research from the AIDS Reagent project (National Institute for Biological Standards and Control, Potters Bar, UK). Alum (aluminum phosphate) was a gift from P. Frezza (Hardis, Naples, Italy). Immune-stimulating complexes containing the Tat protein (80  $\mu$ g/ml) were prepared with a described procedure<sup>4</sup>. ELISA and western blot analysis were done to confirm the co-localization of the immune-stimulating complexes with the Tat protein (data not shown).

**Detection of antibodies against Tat.** Polyvinyl chloride microtiter plates were coated with Tat (100 ng/200  $\mu$ l per well of 0.05 M carbonate buffer, pH 9.6) for 12 h (4 °C) and extensively washed with PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> containing 0.05 % Tween 20 (PBS/Tween). Plasma, 200  $\mu$ l diluted in buffer, was then added to each well in duplicate. Plates were incubated for 90 min (37 °C), washed five times with PBS/Tween and 100  $\mu$ l horseradish peroxidase-conjugated secondary antibody (Sigma) diluted 1:1,000 in PBS/Tween (containing 1% BSA) was added for 90 min (37 °C). After extensive washing of the plates, 100  $\mu$ l peroxidase substrate (ABTS 1 mM, Amersham Pharmacia Biotech, Milan, Italy) was added and the absorbance at 405 nm was measured with a spectrophotometer. A rabbit polyclonal antiserum against Tat, used at serial twofold dilutions (1:200-1:6,400), was the positive control. Monkey preimmune plasma (diluted 1:50 and 1:100) was the negative control. The mean of the negative controls + 3 standard deviations represented the cut-off value. The minimal plasma dilution used was 1:50.



**Neutralization of Tat activity on HIV replication by the rescue assay.** Tat activity was measured by the rescue assay in which the replication of Tat-defective HIV-1 proviruses is induced by serial concentrations of Tat added to HLM-1 cells (HeLa CD4<sup>+</sup> cells containing a Tat-defective HIV-1 provirus) as described<sup>16,17,20</sup>. Growth medium (300  $\mu$ l) containing either preimmune or immune plasma (1:2 dilution) were added to each well (in duplicate). Supernatants were collected 48 h later and p24 Gag content was determined by an antigen capture assay (NEN).

**Lymphoproliferative responses.** PBMCs were purified from citrated peripheral blood on a Ficoll gradient with a standard procedure. PBMCs ( $2 \times 10^5$  in 100  $\mu$ l of growth medium) were plated with 100  $\mu$ l of medium, PHA (2  $\mu$ g/ml; Murex Biotech Limited, Dartford, UK), tetanus toxoid (5  $\mu$ g/ml) or Tat (5  $\mu$ g/ml) each in triplicate wells. After 5 d, 1  $\mu$ Ci <sup>3</sup>H-thymidine was added and the radioactivity in samples was measured 16 h later with a Betaplate (Wallac, Turku, Finland).

**CTL assay.** PBMCs were seeded ( $5 \times 10^5$ /well in 0.5 ml complete medium) in a 24-well plate with Tat (1  $\mu$ g). One day later,  $5 \times 10^4$  PBMCs were incubated for 3 h with Tat (1  $\mu$ g), washed twice and added to the wells containing the PBMCs stimulated previously. On day 2, 2 IU of recombinant human IL-2 (rhIL-2) was added to each well. Half of the supernatant was replaced with medium containing rhIL-2 twice each week. On day 14, cells were collected, counted, resuspended in growth medium containing 1 mM sulfinpyrazone (Sigma) and seeded (96-well round-bottomed plates) at serial twofold dilutions (in duplicate) (effectors). The day before the assay, her-

pesvirus Papio-transformed autologous B lymphocytes<sup>44</sup> were pulsed overnight with or without Tat (4 µg/10<sup>6</sup> cells; targets), labeled with the fluorescence-enhancing ligand bis (acetoxymethyl 2,2':6',2"-terpyridine-6,6'-dicarboxylate (BATDA) according to the manufacturer's instructions (Defila; Wallac, Turku, Finland) (ref. 56), and 5 × 10<sup>3</sup> cells were added to the effectors. After 2 h, 20 µl of supernatants were mixed with 200 µl of Europium solution, and fluorescence was measured after 20 min with a time-resolved fluorescence reader (Victor; Wallac, Turku, Finland). The percent specific lysis was calculated for each effector:target ratio as follows: (test release-spontaneous release)/(maximum release-spontaneous release) × 100. The percent specific lysis against unpulsed autologous B lymphocytes was calculated and subtracted from the percent specific lysis against the Tat-pulsed targets. The assay was considered positive for values exceeding 10%.

**TNF-α production in PBMCs, CD8<sup>+</sup> or CD8<sup>+</sup> T cells after Tat stimulation.** PBMCs were seeded (in duplicate wells) in a 24-well plate in 1 ml complete medium (1 × 10<sup>6</sup> cells/ml) and stimulated with PHA (2 µg/ml) or Tat (5 µg/ml). After 2 days, 100 µl of the cell supernatants was collected, and TNF-α production was measured by ELISA as suggested by the manufacturer (Cytoscreen Monkey for TNF-α; Biosource, Camarillo, California).

To determine the cell source of TNF-α production, unfractionated PBMCs were seeded in a 6-well plate in 4 ml complete medium (2 × 10<sup>6</sup> cells/ml) and stimulated with Tat (5 µg/ml). After 2 d, cells were washed, counted and separated into CD8<sup>+</sup> and CD8<sup>+</sup> cells using Dynabeads (Dyna, Oslo, Norway) following the manufacturer's instructions. The purity of the cell populations was evaluated by three-color FACS analysis using monoclonal antibodies against CD3, CD4 and CD8. CD8<sup>+</sup> and CD8<sup>+</sup> cells were separately plated (5 × 10<sup>4</sup> cells/well) in a total volume of 200 µl in a U-bottomed 96-well plate in the presence of 2 U/ml of IL-2. Supernatants (100 µl) from the two different cell populations were collected after an additional 24 h, and TNF-α production was evaluated by ELISA.

**Skin tests for tetanus toxoid and Tat.** PBS containing 0.1% BSA (150 µl) alone (negative control) or with tetanus toxoid (7 µg) or Tat (5 or 1 µg) was injected intradermally on a shaved area of the upper back. Monkeys were assessed at 24, 48 and 72 h after this injection. The test was considered strongly positive (++) or positive (+) in the presence of induration and erythema with a diameter of ≥5 mm or 1–4 mm, respectively. Erythema without induration or erythema < 1 mm were considered weakly positive (±) or negative (-), respectively.

**Generation of SHIV-89.6P virus stock, and *in vivo* and *in vitro* titration.** To prepare the virus stock, the parental SHIV-89.6P (ref. 47) (obtained from N. Letvin, Harvard Medical School, Boston, Massachusetts) was used to infect a cynomolgus macaque. At day 14 after infection, the monkey was killed and total or CD8<sup>+</sup> T cell-depleted cells obtained from spleen and lymph nodes were stimulated *in vitro* with PHA (2 µg/ml) and cultured in RPMI containing 15% FCS and 50 IU/ml of rIL-2. Supernatants showing reverse transcriptase activity > 50,000 cpm/ml were pooled, centrifuged at 912g for 20 min (4 °C), clarified at 3,200g for 30 min (4 °C). The supernatant was then complemented with 10% of human serum ABO and frozen at -152 °C. Frozen vials (0.5 ml) were then tested to determine the tissue culture infectious dose (TCID<sub>50</sub>) using CEMx174 cells, C8166 cells and PBMCs from four naive macaques.

To determine the MID<sub>50</sub>, the viral stock was titrated in eight monkeys by intravenous inoculum of serial virus dilutions (5 × 10<sup>-1</sup> to 5 × 10<sup>-6</sup>). Infection was monitored by antibody response, plasma antigenemia and viremia, virus isolation, proviral DNA and CD4 T-cell counts. All these parameters were in the same range of those published<sup>47</sup> and those from six monkeys inoculated in our lab with the original virus stock from rhesus macaques. The MID<sub>50</sub> was then calculated according to Reed and Muench.

**Quantitation of the SHIV RNA copies in plasma.** The quantitation of plasma SHIV-89.6P RNA copies was done by the Chiron Corporation in the Chiron Diagnostics Reference Testing Laboratory (Amsterdam, the Netherlands) with a branched DNA signal amplification assay recognizing the *pol* region of the SIVmac strains as described<sup>49</sup>. The cut-off value was 1,500 RNA copies/ml; however, values less than 3,000 RNA copies/ml were not always reproducible.

**Quantitation of SIV proviral copies.** DNA was extracted from whole blood (QIAamp Blood Kit; Qiagen) and tested for amplification of the β-globin gene as described<sup>50</sup>. To determine the number of SIV proviral copies, semi-quantitative DNA PCR was done to amplify a 496-bp region of the *gag* gene of SIV<sub>mac251</sub> with primers and methodologies described<sup>5</sup>.

**Virus isolation and cell associated viral load (cytoviremia).** For virus isolation, CD8<sup>+</sup> T cell-depleted PBMCs (3 × 10<sup>6</sup>) were co-cultured with 1 × 10<sup>4</sup> CEM x 174 cells in the presence of PHA (2 µg/ml) for 2–3 d and cultured for 30–40 d in RPMI 1640 containing 10% FCS, antibiotics and rIL-2 (50 IU/ml). The titer of p27 SIV-Gag antigen was determined twice a week.

For cytoviremia, serial twofold dilutions of CD8-depleted PBMCs (1 × 10<sup>4</sup> to 4.8 × 10<sup>4</sup> cells/well, in duplicate), were co-cultured with CEMx174 cells (1 × 10<sup>4</sup> cells/well) and assessed for the presence of p27 antigen production on day 12. The 50% endpoints were calculated with the method of Reed and Muench, and results are expressed as the number of infected cells per 1 × 10<sup>6</sup> PBMCs.

**Determination of antibodies against HIV-2/SIV and HIV-1 Env in plasma and *in vitro* antibody production.** Antibody titers against SIV were determined by end-point dilution using an HIV-2 ELISA assay (Elavia, Ac- Ab-Ak II kit; Diagnostic Pasteur, Paris, France). Antibodies against HIV-1 Env in plasma were determined by HIV-1 ELISA assay (HIV-1/HIV-2 Third Generation Plus; Abbott, Chicago Illinois).

For the *in vitro* antibody production assay, 2 × 10<sup>6</sup> PBMCs/well were seeded onto a 24-well culture plate with 2 µg/ml of PWM (Sigma) (ref. 59) and incubated for 7 d. Supernatants were then collected, centrifuged (3,200g for 10 min) and tested for HIV-1 Env antibodies.

**Detection of p27 'antigenemia'.** Levels of p27 SIV-Gag protein were measured in plasma by using an antigen capture ELISA assay (Innotest, Innogenetics, Zwijndrecht, Belgium) with a limit of detection of 20 pg/ml.

**CD4<sup>+</sup> and CD8<sup>+</sup> T-cell counts.** Citrated peripheral blood (80–100 µl) was stained with phycoerythrin (PE)-conjugated monoclonal antibodies against CD4 (Biosource, Camarillo, California) and with peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibodies against CD8 (Becton-Dickinson, Mountain View, California) and analyzed with a FACScan cytometer and software (Becton-Dickinson, Mountain View, California) as described<sup>51</sup>. Isotype-matched PE- and PerCP-labeled antibodies were the negative controls. Absolute cell numbers were calculated from the blood cell counts.

#### Acknowledgments

We thank all personnel responsible of the animal facility; C. Sgadari, D. Negri, I. Macchia, Z. Michellini, M. Barbisin, E. Salvi and C. Rovetto (Laboratory of Virology, Istituto Superiore di Sanità, Rome, Italy) for technical help; P. Markham and B.C. Nair (Advanced BioScience laboratories, Kensington, Maryland) for Tat protein production; A. Lippe and F.M. Regini for editorial assistance; and G. Benagliano (Director General, ISS, Rome, Italy) for his support to the study. This work was supported by Italian grants from the ISS, Rome, Italy, IX AIDS Project and from the Associazione Nazionale per la Lotta contro l'AIDS (ANLAIDS).

RECEIVED 4 MARCH; ACCEPTED 25 MARCH 1999

1. Almond, N.M. & Heeney, J.L. AIDS vaccine development in primate models. *AIDS* 12, 133–140 (1998).
2. Burton, D.R. & Moore, J.P. Why do not have an HIV vaccine and how can we make one? *Nature Med.* 4, 495–498 (1998).
3. Berman, P.W. et al. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* 345, 622–625 (1990).
4. Girard, M. et al. Immunization of chimpanzees confers protection against challenge with human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 88, 542–546 (1991).
5. Stott, E.J. et al. Evaluation of a candidate human immunodeficiency virus type 1 (HIV-1) vaccine in macaques: effects of vaccination with HIV-1 gp120 on subsequent challenge with heterologous simian immunodeficiency virus-HIV-1 virus. *J. Gen. Virol.* 79, 423–432 (1998).

6. Kestler, H.D. *et al.* Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. *Cell* 65, 651-662 (1991).
7. Daniel, M.D., Kirchhoff, F., Czajak, S.C., Sehgal, P.K., & Desrosiers, R.C. Protective effects of a live attenuated SIV vaccine with a deletion in the *nef* gene. *Science* 258, 1938-1941 (1992).
8. Tittü, F. *et al.* Live-attenuated simian immunodeficiency virus prevents super-infection by cloned SIVmac251 in Cynomolgus monkeys. *J. Gen. Virol.* 78, 2529-2539 (1997).
9. Whatmore, A.M. *et al.* Repair and evolution of *nef* in vivo modulates Simian Immunodeficiency Virus virulence. *J. Virol.* 69, 5117-5123 (1995).
10. Baba, T.W. *et al.* Pathogenicity of live attenuated SIV after mucosal infection of neonatal macaques. *Science* 267, 1820-1825 (1995).
11. Ezzell, C. The monkey's got AIDS: what now for live AIDS vaccine? *J. NIH Res.* 9, 21-22 (1997).
12. Arya, S.K., Guo, C., Josephs, S.F. & Wong-Staal, F. The trans-activator gene of human T-lymphotropic virus type III (HTLV-III). *Science* 229, 69-73 (1985).
13. Fisher, A.G. *et al.* The trans-activator gene of HTLV-III is essential for virus replication. *Nature* 320, 367-371 (1986).
14. Chang, H.-K., Gallo, R.C. & Ensoli, B. Regulation of cellular gene expression and function by the human immunodeficiency virus type 1 Tat protein. *J. Biomed. Sci.* 2, 189-202 (1995).
15. Ensoli, B. *et al.* Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature* 345, 84-87 (1990).
16. Ensoli, B. *et al.* Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J. Virol.* 67, 277-287 (1993).
17. Chang, H.-K., Samaniego, F., Nair, B.C., Buonaguro, L. & Ensoli, B. HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. *AIDS* 11, 1421-1431 (1997).
18. Westendorp, M.O. *et al.* Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp 120. *Nature* 375, 497-500 (1995).
19. Frankel, A.D. & Pabo, C.O. Cellular uptake of the Tat protein from human immunodeficiency virus. *Cell* 55, 1189-1193 (1988).
20. Barillari, G. *et al.* Effect of cytokines from activated immune cells on vascular cell growth and HIV-1 gene expression. Implications for AIDS-Kaposi's sarcoma pathogenesis. *J. Immunol.* 149, 3727-3734 (1992).
21. Huang, L., Bosh, L., Hofmann, W., Sodroski, J. & Pardee, A.B. Tat protein induces human immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with both macrophage-tropic and T-lymphotropic HIV-1 strains. *J. Virol.* 72, 8952-8960 (1998).
22. Li, C.J. *et al.* Tat protein induces self-perpetuating permissivity for productive HIV-1 infection. *Proc. Natl. Acad. Sci. USA* 94, 8116-8120 (1997).
23. Viscidi, R.P., Mayur, K., Lederer, H.M. & Frankel, A.D. Inhibition of antigen-induced lymphocyte proliferation by Tat protein from HIV-1. *Science* 246, 1606-1608 (1989).
24. Barillari, G., Gendelman, R., Gallo, R.C. & Ensoli, B. The Tat protein of human immunodeficiency virus type-1, a growth factor for AIDS Kaposi's sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence. *Proc. Natl. Acad. Sci. USA* 90, 7941-7945 (1993).
25. Subramanyam, M., Gutheil, W.G., Bachovchin, W.W. & Huber, B.T. Mechanism of HIV-1 Tat induced inhibition of antigen-specific T cell responsiveness. *J. Immunol.* 150, 2544-2553 (1993).
26. Zagury, J.F. *et al.* Interferon alpha and Tat involvement in the immunosuppression of uninfected T cells and C-C chemokine decline in AIDS. *Proc. Natl. Acad. Sci. USA* 95, 3851-3856 (1998).
27. Ensoli, B. *et al.* Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. *Nature* 371, 674-680 (1994).
28. Li, C.J., Friedman, D.J., Wang, C., Metelev, V. & Pardee, A.B. Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. *Science* 268, 429-431 (1995).
29. Zauli, G. *et al.* Pleiotropic effects of immobilized versus soluble recombinant HIV-1 Tat protein on CD3-mediated activation, induction of apoptosis, and HIV-1 long terminal repeat transactivation in purified CD4<sup>+</sup> T lymphocytes. *J. Immunol.* 157, 2216-2224 (1996).
30. Zagury, J.F. *et al.* Mode of AIDS immunopathogenesis based on the HIV-1 gp120 and Tat-induced dysregulation of uninfected immune cells. *Cell Pharmacol.* 3, 123-128 (1996).
31. Relis, P. *et al.* Speed of progression to AIDS and degree of antibody response to accessory gene products of HIV-1. *J. Med. Virol.* 30, 163-168 (1990).
32. Rodman, T.C., To, S.E., Hashish, H. & Manchester, K. Epitopes for natural antibodies of human immunodeficiency virus (HIV)-negative (normal) and HIV-positive sera are coincident with two key functional sequences of HIV Tat protein. *Proc Natl Acad Sci. USA* 90, 7719-7723 (1993).
33. Re, C. *et al.* Effect of antibody to HIV-1 Tat protein on viral replication in vitro and progression of HIV-1 disease in vivo. *J. Acquir. Immune Defic. Syndr. Hum. Retrovir.* 10, 408-416 (1993).
34. Zagury, J.F. *et al.* Antibodies to the HIV-1 Tat protein correlated with nonprogression to AIDS: a rationale for the use of Tat toxoid as an HIV-1 vaccine. *J. Hum. Virol.* 4, 282-292 (1998).
35. Venet, A., Bourgault, I., Aubertin, A.M., Kiény, M.P. & Levy, J.P. Cytotoxic T lymphocyte response against multiple simian immunodeficiency virus (SIV) proteins in SIV-infected macaques. *J. Immunol.* 148, 2899-2908 (1992).
36. Van Baalen, C.A. *et al.* HIV-1 Rev and Tat specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS. *J. Gen. Virol.* 78, 1913-1918 (1997).
37. Froebel, K.S. *et al.* Cytotoxic T lymphocyte activity in children infected with HIV. *AIDS Res. Hum. Retrovir.* 2, 83-88 (1994).
38. Fawell, S. *et al.* Tat-mediated delivery of heterologous proteins into cells. *Proc. Natl. Acad. Sci. USA* 91, 664-668 (1994).
39. Kim, D.T. *et al.* Introduction of soluble proteins into the MHC class I pathway by conjugation to an HIV tat peptide. *J. Immunol.* 159, 1666-1668 (1997).
40. Human Retroviruses and AIDS 1995. *A Compilation and Analysis of Nucleic Acids and Amino Acid Sequences* (eds. Korber, B. *et al.*) II-A-55, 56 (Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, New Mexico, 1995).
41. Goldstein, G. HIV-1 Tat protein as a potential AIDS vaccine. *Nature Med.* 1, 960-964 (1995).
42. Davis, D. *et al.* A recombinant prime, peptide boost vaccination strategy can focus the immune response on to more than one epitope even though these may not be immunodominant in the complex immunogen. *Vaccine* 15, 1661-1669 (1997).
43. Perez, C. *et al.* A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. *Cell* 63, 251-258 (1990).
44. Jassoy, C. *et al.* Human immunodeficiency virus type 1-specific cytotoxic T lymphocytes release gamma interferon, tumor necrosis factor alpha (TNF $\alpha$ ), and TNF $\beta$  when they encounter their target antigens. *J. Virol.* 67, 2844-2852 (1993).
45. Ratner, A. & Clark, W.R. Role of TNF- $\alpha$  in CD8<sup>+</sup> cytotoxic T lymphocyte-mediated lysis. *J. Immunol.* 150, 4303-4314 (1993).
46. Ando, K. *et al.* Perforin, Fas/Fas ligand, and TNF- $\alpha$  pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. *J. Immunol.* 158, 5283-5291 (1997).
47. Reimann, K.A. *et al.* A chimeric simian/human immunodeficiency virus expressing a primary patients human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. *J. Virol.* 70, 6922-6928 (1996).
48. Lamhamedi-Cherradi, S. *et al.* Qualitative and quantitative analysis of human cytotoxic T-lymphocyte responses to HIV-1 proteins. *AIDS* 6, 1249-1258 (1992).
49. Rinaldo, C.R. *et al.* Anti-HIV type 1 cytotoxic T lymphocyte effector activity and disease progression in the first 8 years of HIV type 1 infection of homosexual men. *AIDS Res. Hum. Retrovir.* 11, 481-489 (1995).
50. Ogg, G.S. *et al.* Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279, 2103-2106 (1998).
51. Buonaguro, L. *et al.* Heteroduplex mobility assay and phylogenetic analysis of V3 region sequences of human immunodeficiency virus type 1 isolates from Gulu, northern Uganda. The Italian-Ugandan Cooperation AIDS Program. *J. Virol.* 69, 7971-7981 (1995).
52. Calarota, S. *et al.* Cellular cytotoxic response induced by DNA vaccination in HIV-1 infected patients. *Lancet* 351, 1320-1325 (1998).
53. Gringeri, A. *et al.* Safety and immunogenicity of HIV-1 Tat Toxoid in Immunocompromised HIV-1 Infected Patients. *J. Hum. Virol.* 1, 293-298 (1998).
54. Caselli, E. *et al.* DNA Immunization with HIV-1 tat Mutated in the Transactivation Domain Induces Humoral and Cellular Immune Responses against Wild-Type TAT. *J. Immunol.* (in the press).
55. Chen, Z.W. *et al.* Cytotoxic T lymphocytes do not appear to select for mutations in an immunodominant epitope of simian immunodeficiency. *J. Immunol.* 149, 4060-4066 (1992).
56. Lövgren, J. & Blomberg, K. Simultaneous measurement of NK cell cytotoxicity against two target cell lines labelled with fluorescent lanthanide chelates. *J. Immunol. Methods* 173, 119-125 (1994).
57. Sodora, D.L., Lee, F., Dailey, P.J. & Marx, P.A. A genetic and viral load analysis of the simian immunodeficiency virus during the acute phase in macaques inoculated by the vaginal route. *AIDS Res. Hum. Retroviruses* 14, 171-181 (1998).
58. Saiki, R.K. *et al.* Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350-1354 (1985).
59. Fiore, J.R. *et al.* Pokeweed mitogen-stimulated peripheral blood mononuclear cells from at-risk seronegative subjects produce in vitro HIV-1-specific antibodies. *AIDS* 5, 1034-1036 (1991).

## Reduction of Simian-Human Immunodeficiency Virus 89.6P Viremia in Rhesus Monkeys by Recombinant Modified Vaccinia Virus Ankara Vaccination

DAN H. BAROUCH,<sup>1\*</sup> SAMPA SANTRA,<sup>1</sup> MARCELO J. KURODA,<sup>1</sup> JÖRN E. SCHMITZ,<sup>1</sup> RONALD PLISHKA,<sup>2</sup> ALICIA BUCKLER-WHITE,<sup>2</sup> ALICIA E. GATIAN,<sup>3</sup> REBEKAH ZIN,<sup>3</sup> JAE-HWAN NAM,<sup>4</sup> LINDA S. WYATT,<sup>4</sup> MICHELLE A. LIFTON,<sup>1</sup> CHRISTINE E. NICKERSON,<sup>1</sup> BERNARD MOSS,<sup>4</sup> DAVID C. MONTEFIORE,<sup>3</sup> VANESSA M. HIRSCH,<sup>2</sup> AND NORMAN L. LETVIN<sup>1</sup>

*Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215<sup>1</sup>;  
Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Rockville, Maryland 20852<sup>2</sup>;  
Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892<sup>3</sup>;  
and Duke University Medical Center, Durham, North Carolina 27710<sup>4</sup>*

Received 8 December 2000 / Accepted 3 March 2001

Since cytotoxic T lymphocytes (CTLs) are critical for controlling human immunodeficiency virus type 1 (HIV-1) replication in infected individuals, candidate HIV-1 vaccines should elicit virus-specific CTL responses. In this report, we study the immune responses elicited in rhesus monkeys by a recombinant poxvirus vaccine and the degree of protection afforded against a pathogenic simian-human immunodeficiency virus SHIV-89.6P challenge. Immunization with recombinant modified vaccinia virus Ankara (MVA) vectors expressing SIVmac239 gag-pol and HIV-1 89.6 env elicited potent Gag-specific CTL responses but no detectable SHIV-specific neutralizing antibody (NAb) responses. Following intravenous SHIV-89.6P challenge, sham-vaccinated monkeys developed low-frequency CTL responses, low-titer NAb responses, rapid loss of CD4<sup>+</sup> T lymphocytes, high-setpoint viral RNA levels, and significant clinical disease progression and death in half of the animals by day 168 postchallenge. In contrast, the recombinant MVA-vaccinated monkeys demonstrated high-frequency secondary CTL responses, high-titer secondary SHIV-89.6-specific NAb responses, rapid emergence of SHIV-89.6P-specific NAb responses, partial preservation of CD4<sup>+</sup> T lymphocytes, reduced setpoint viral RNA levels, and no evidence of clinical disease or mortality by day 168 postchallenge. There was a statistically significant correlation between levels of vaccine-elicited CTL responses prior to challenge and the control of viremia following challenge. These results demonstrate that immune responses elicited by live recombinant vectors, although unable to provide sterilizing immunity, can control viremia and prevent disease progression following a highly pathogenic AIDS virus challenge.

A safe and effective human immunodeficiency virus type 1 (HIV-1) vaccine is urgently needed to control the worldwide HIV-1 epidemic. A number of recent studies have demonstrated the importance of virus-specific CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) in controlling HIV-1 replication in humans and simian immunodeficiency virus (SIV) replication in rhesus monkeys (18, 26, 27, 36). It is therefore widely believed that HIV-1 vaccine candidates should elicit potent virus-specific CTL responses in addition to neutralizing antibody (NAb) responses.

Live, attenuated virus vaccines have been shown to generate CTL and NAb responses capable of controlling a number of pathogenic viral challenges (10, 40, 41). However, significant safety concerns regarding this approach remain. Live, attenuated SIV vaccines have been shown to induce AIDS in neonatal and adult macaques (4, 5). More importantly, humans infected with *nef*-deleted HIV-1 have been reported to develop immunodeficiency and clinical disease (11, 14, 22).

Other vaccine strategies capable of eliciting virus-specific

CTL responses are therefore being evaluated. Approaches that have generated considerable interest include plasmid DNA and recombinant live vectors. We have recently reported that plasmid DNA vaccination elicited high-frequency CTL responses that reduced setpoint viremia following an SIVsmE660 challenge in rhesus monkeys (12). We have also demonstrated that cytokine-augmented DNA vaccination elicited potent immune responses that effectively controlled viremia and prevented clinical disease progression following a pathogenic simian-human immunodeficiency virus SHIV-89.6P challenge (6, 7).

It remains to be determined whether other vaccination modalities, in particular live recombinant vectors, will provide a similar level of protection in monkeys challenged with the highly pathogenic virus SHIV-89.6P (32–34). A number of recombinant live poxviruses have been evaluated for their utility as HIV-1 vaccine candidates. Safety concerns regarding vaccinia virus (31) have led to the development of a number of attenuated poxviruses as vaccine vectors, including NYVAC, fowlpox, canarypox, and modified vaccinia virus Ankara (MVA) (8, 16, 28, 29, 37, 38). MVA is an attenuated form of vaccinia virus that has undergone 570 passages in primary chicken embryo fibroblasts and has genomic deletions that reduce its pathogenicity (23). We have recently reported that a recombinant MVA/gag-pol vaccine elicits SIV-specific CTL responses in rhesus monkeys (37). Following a pathogenic

\* Corresponding author. Mailing address: Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Research East Room 113, 330 Brookline Ave., Boston, MA 02215. Phone: (617) 667-2042. Fax: (617) 667-8210. E-mail: dan\_barouch@hotmail.com.

STVsmE660 challenge, secondary CTL responses were detected associated with a partial control of viremia (38). In another study, vaccination with recombinant MVA/*env*, MVA/*gag-pol*, or MVA/*gag-pol-env* constructs reduced plasma viremia and increased survival following an STVsmE660 challenge (28, 29).

In the present study, we investigate the ability of recombinant MVA vectors expressing STV *gag-pol* and HIV-1 *env* derived from the primary patient isolate 89.6 to elicit CTL and NAb responses in rhesus monkeys. We also assess the protection afforded by these immune responses against a highly pathogenic SHIV-89.6P challenge.

#### MATERIALS AND METHODS

**Construction of recombinant MVA vectors.** Open reading frames of STVmac239 *gag-pol* and HIV-1 89.6 *env* truncated at amino acid 738 were inserted adjacent to the modified HS promoter in the previously described plasmid transfer vectors pLW-9 and pLW-17, respectively (42, 43). Recombinant MVA/*gag-pol* and MVA/*env* vectors were each produced by homologous recombination, identified by immunostaining of live, infected cell foci, and clonally isolated. The purity of each recombinant virus was assessed by PCR and immunostaining. Expression of the recombinant proteins was determined by radioimmunoprecipitation. The production of Gag particles and surface expression and fusion competence of the expressed Env proteins were demonstrated.

**Vaccination and challenge of rhesus monkeys.** Eight *Mamu-A\*01*-positive rhesus monkeys were selected for inclusion in this study (20). The monkeys were immunized intramuscularly with  $10^6$  PFU of either control nonrecombinant MVA ( $n = 4$ ) or recombinant MVA vectors expressing STV *gag-pol* and HIV-1 89.6 *env* at weeks 0, 4, and 21. The monkeys were challenged at week 27 with a 1:500 dilution (estimated 100 50% monkey infective doses [MID<sub>50</sub>]) of the uncloned cell-free SHIV-89.6P stock (33, 34) by the intravenous (i.v.) route. Monkeys were maintained in accordance with National Institutes of Health and Harvard Medical School guidelines.

**Tetramer staining.** Tetramer staining was performed with freshly isolated peripheral blood mononuclear cells (PBMC) from EDTA-anticoagulated whole blood specimens as described (3, 21). Briefly, soluble tetrameric Mamu-A\*01 complexes folded around the STV Gag p11C epitope (CTPYDINQM) (1, 24) were prepared. One microgram of phycoerythrin-labeled tetrameric Mamu-A\*01/p11C complexes was used in conjunction with fluorescein isothiocyanate-labeled anti-human CD8 $\alpha$  (Leu2 $\alpha$ ; Becton Dickinson), phycoerythrin-Texas Red (ECD)-labeled anti-human CD8 $\beta$  (2ST8-SH7; Becton Coulter), and allophycocyanin-labeled anti-rhesus monkey CD3 (FN18) monoclonal antibodies to stain p11C-specific CD8 $^{+}$  T cells. A total of 100  $\mu$ l of whole blood from the vaccinated or control monkeys was directly stained with these reagents, lysed, washed, and fixed. Samples were analyzed by four-color flow cytometry with a Becton Dickinson FACS Calibur system, and gated CD3 $^{+}$  CD8 $\alpha\beta$  $^{+}$  T cells were examined for staining with tetrameric Mamu-A\*01/p11C complexes.

**CTL assays.** Functional chromium release cytotoxicity assays were performed as described (6). Briefly,  $5 \times 10^6$  washed PBMC from rhesus monkeys were cultured in the presence of 10  $\mu$ g of p11C peptide (CTPYDINQM)/ml (1, 24). On day 3 of culture, 20 U of human recombinant interleukin 2 (Hoffmann-La Roche)/ml was added. On day 12 of culture, peptide-stimulated PBMC were centrifuged over Ficoll (Ficoll-Paque) and assessed as effectors in standard 4-h  $^{51}$ Cr-release assays containing  $10^4$  target cells/well. Autologous B-lymphoblastoid cell lines pulsed with 1  $\mu$ g of p11C peptide or p11B control peptide (ALSEGCTPYDIN)/ml and labeled overnight with  $^{51}$ Cr (100  $\mu$ Ci/ml) were used as targets. To measure spontaneous release of  $^{51}$ Cr, target cells were incubated with 100  $\mu$ l of medium, and for maximum release target cells were incubated with 100  $\mu$ l of 2% Triton X-100. Percent lysis was calculated as follows: [(experimental release - spontaneous release)/(maximum release - spontaneous release)]  $\times$  100.

**Neutralizing antibody assays.** Determination of antibody titers capable of neutralizing SHIV-89.6 and SHIV-89.6P was performed as described (9). Briefly, reduction of virus-induced cytopathic killing of MT-2 cells was measured by Finter's neutral red that is taken up by viable cells. A total of 50  $\mu$ l of cell-free virus containing 500 50% tissue culture infective doses grown in human PBMC was added to multiple dilutions of test plasma in 100  $\mu$ l of growth media in triplicate. These mixtures were incubated for 1 h before the addition of  $5 \times 10^4$  MT-2 cells. Infection led to extensive syncytium formation and virus-induced cell killing in 4 to 6 days in the absence of neutralizing antibodies. Neutralization

titer was calculated as the reciprocal dilution of plasma required to protect 50% of cells from virus-induced killing.

**CD4 $^{+}$  T-lymphocyte counts and viral RNA levels.** CD4 $^{+}$  T-lymphocyte counts were determined by multiplying the total lymphocyte count by the percentage of CD3 $^{+}$  CD4 $^{+}$  T cells assessed by flow cytometry. Plasma viral RNA levels were measured by a real-time reverse transcriptase PCR amplification assay with a detection limit of 500 copies/ml as described (17) using *gag* primers and probes (39).

**Statistical analyses.** Statistical analyses were performed with GraphPad Prism version 2.01 (GraphPad Software, Inc.). CD4 $^{+}$  T-lymphocyte counts and viral RNA levels were compared between groups by two-sided Wilcoxon rank sum tests. Day 70 setpoint values were chosen in order to analyze a complete data set prior to the death of any animals. Correlation of prechallenge vaccine-elicited CTLs and day 70 postchallenge setpoint viral RNA levels was assessed by a two-sided Spearman rank correlation test. In all cases, a *P* value of <0.05 was considered significant.

#### RESULTS

**Vaccine trial design.** Eight rhesus monkeys (*M. mulatta*) expressing the major histocompatibility complex class I allele *Mamu-A\*01* were selected for inclusion in this study (20). These animals were immunized with the control nonrecombinant MVA ( $n = 4$ ) or recombinant MVA vaccines expressing STV *gag-pol* and HIV-1 *env* derived from the primary patient R5/X4 dual-tropic isolate 89.6 ( $n = 4$ ). Animals received  $10^6$  PFU intramuscularly of control or recombinant MVA vectors at weeks 0, 4, and 21. At week 27, all eight animals were challenged i.v. with SHIV-89.6P. This highly pathogenic virus was derived by *in vivo* passage of the nonpathogenic virus SHIV-89.6 and has been shown to cause rapid CD4 $^{+}$  T-lymphocyte loss and clinical AIDS in the majority of naïve rhesus monkeys (7, 32–34).

**Vaccine-elicited immune responses.** Staining CD8 $^{+}$  T cells with tetrameric MHC class I-peptide complexes followed by analysis by flow cytometry has proven to be an accurate method for quantitating epitope-specific CTLs in freshly isolated whole-blood specimens without the need for *in vitro* lymphocyte stimulation (3, 21). CTL responses specific for the Mamu-A\*01-restricted immunodominant STV Gag p11C epitope (CTPYDINQM) (1, 24) were measured by both tetramer staining and functional chromium release cytotoxicity assays. As shown in Fig. 1, p11C-specific CTLs were detected by tetramer staining in all vaccinated animals after the initial immunization. Higher levels were observed 1 week after the week 4 and week 21 boost immunizations, reaching a maximum of 0.2 to 0.8% of circulating CD3 $^{+}$  CD8 $^{+}$  T cells. Levels of CTLs following the second and third immunizations were comparable, consistent with the findings in our previous study of CTL responses elicited by recombinant MVA vectors in rhesus monkeys (37, 38). Following each boost immunization, there was a rapid expansion of p11C-specific CTLs followed by a rapid decline to steady-state plateau levels of 0.1 to 0.3% of circulating CD8 $^{+}$  T cells that persisted over time. Tetramer staining specific for the subdominant HIV-1 Env p41A epitope (YAPPIISGQI) (13) was only detected in one animal (H507), and no tetramer staining specific for p11C or p41A was observed in the monkeys that received the control MVA (data not shown). As shown in Table 1, functional chromium release cytotoxicity assays confirmed these tetramer staining data. No NAb responses specific for SHIV-89.6 or SHIV-89.6P (<1:20 titer) were detectable in the control or vaccinated animals at peak immunity or prior to challenge (data not shown).



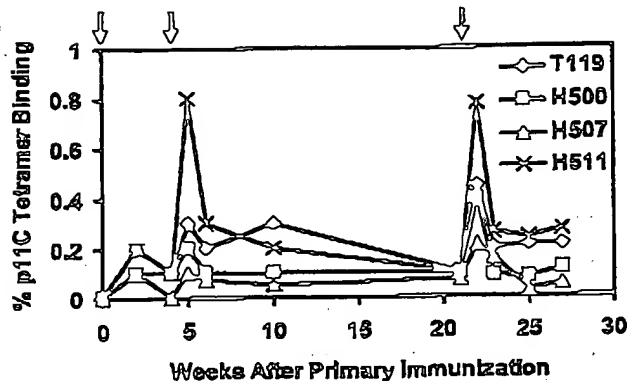


FIG. 1. Vaccine-elicited CTL responses. *Mamu-A\*01*-positive monkeys were immunized at weeks 0, 4, and 21 with recombinant MVA constructs expressing SIV *gag-pol* and HIV-1 89.6 *env*. Vaccino-elicited CD8<sup>+</sup> T-cell responses specific for the immunodominant SIV Gag p11C (CTPYDINQM) epitope (1, 24) were measured by tetramer staining of freshly isolated PBMC (3, 21). The percent CD3<sup>+</sup> CD8<sup>+</sup> T cells that bound the Mamu-A\*01/p11C tetramer is shown. Arrows indicate times of immunization.

Immune responses following challenge. Six weeks after the final boost immunization, all eight animals were challenged i.v. with 100 MID<sub>50</sub> of cell-free SHIV-89.6P. All animals were infected by this highly pathogenic viral challenge. As shown in Fig. 2, the control monkeys developed primary p11C-specific CTL responses, reaching a maximum of 0.2 to 2% of circulating CD8<sup>+</sup> T cells on day 14 after challenge. In contrast, the vaccinated monkeys developed higher secondary p11C-specific CTL responses, reaching a maximum of 7 to 20% of circulating CD8<sup>+</sup> T cells on day 14 after challenge. As shown in Table 2, the results of functional chromium release cytotoxicity assays confirmed these tetramer staining data.

NAb responses specific for both SHIV-89.6 and SHIV-89.6P were assessed in MT-2 cell-killing assays (9). As shown in Fig. 3A, no SHIV-89.6-specific NAb were detected in the plasma

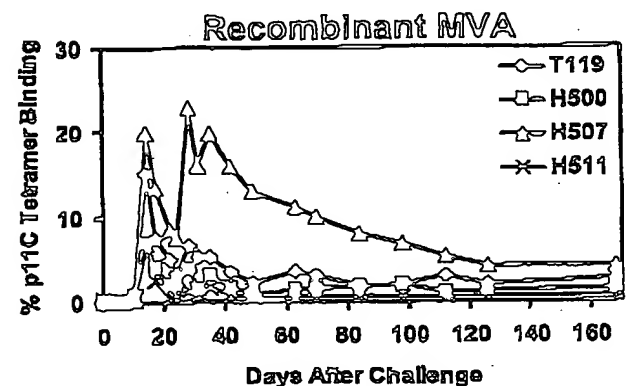
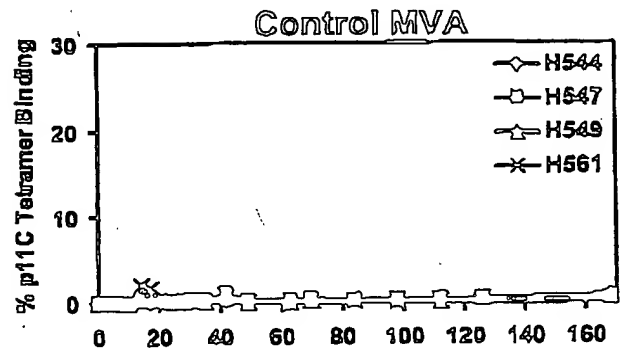


FIG. 2. Secondary CTL responses following challenge. Monkeys were challenged with SHIV-89.6P by the i.v. route on day 0. CD8<sup>+</sup> T-cell responses specific for the SIV Gag p11C epitope were determined by tetramer staining of freshly isolated PBMC at multiple time points (3, 21). The percent CD3<sup>+</sup> CD8<sup>+</sup> T cells that bound the Mamu-A\*01/p11C tetramer is shown.

of the control animals, except for a low titer in monkey H547 on day 70 after challenge. In contrast, SHIV-89.6-specific NAb were detected in the plasma of two vaccinated animals on day 14 after challenge, and high-titer NAb (1,350 to 10,804) were observed in the plasma of all four vaccinated animals on day 21 following challenge. This rapid evolution of high-titer NAb is consistent with a secondary SHIV-89.6-specific NAb response that was primed by the vaccine.

Since NAb generated by SHIV-89.6 infection exhibit poor cross-neutralizing activity against SHIV-89.6P (9, 25), the vaccine expressing HIV-1 Env 89.6 would not be expected to prime for SHIV-89.6P-specific NAb. As shown in Fig. 3B, only two control monkeys (H544 and H547) developed SHIV-89.6P-specific NAb by day 42 after challenge. Surprisingly, all four vaccinated monkeys developed SHIV-89.6P-specific NAb between days 21 and 42 after challenge. The six animals that developed detectable SHIV-89.6P-specific NAb had similar peak titers.

CD4 counts, viral RNA levels, and clinical disease progression. As shown in Fig. 4, the control monkeys developed a rapid and profound loss of CD4<sup>+</sup> T lymphocytes between days 7 and 21 after challenge. Monkeys H549 and H561 demonstrated a complete loss of their CD4<sup>+</sup> T lymphocytes, whereas H544 and H547 had significant but incomplete losses of their

TABLE 1. Vaccine-elicited CTL responses<sup>a</sup>

Vaccination and monkey	Tetramer binding		Functional cytotoxicity (stimulated PBMC)
	Fresh PBMC	Stimulated PBMC	
Control MVA			
H544	0.0	0	0
H547	0.0	0	1
H549	0.0	0	1
H561	0.0	0	0
Recombinant MVA			
T119	0.5	17	37
H500	0.4	4	26
H507	0.2	39	61
H511	0.8	5	30

<sup>a</sup> SIV Gag p11C-specific CD8<sup>+</sup> T-cell responses as measured by tetramer staining of freshly isolated and peptide-stimulated PBMC and chromium release functional cytotoxicity assays at week 22, which is 1 week following the third immunization. The percent CD3<sup>+</sup> CD8<sup>+</sup> cells that bind tetramer is shown for the tetramer assay. The percent specific lysis at a 5:1 effector-to-target ratio is shown for the cytotoxicity assays.

TABLE 2. CTL responses following challenge\*

Vaccination and monkey	Tetramer binding		Functional cytotoxicity (stimulated PBMC)
	Fresh PBMC	Stimulated PBMC	
Control MVA			
H544	0.1	2	2
H547	0.1	1	0
H549	0.7	19	22
H561	1.7	8	11
Recombinant MVA			
T119	7.5	48	29
H500	3.9	49	37
H507	13.5	59	34
H511	2.5	42	28

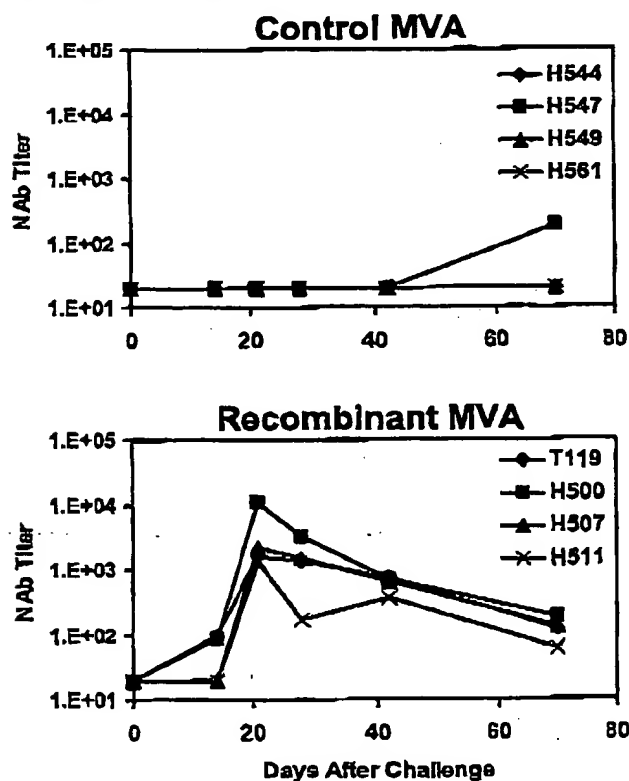
\* SHV Gag p11C-specific CD8<sup>+</sup> T-cell responses as measured by tetramer staining of freshly isolated and peptide-stimulated PBMC and chromium release functional cytotoxicity assays at day 17 after challenge. The percent CD3<sup>+</sup> CD8<sup>+</sup> cells that bind tetramer is shown for the tetramer assays. The percent specific lysis at a 5:1 effector-to-target ratio is shown for the cytotoxicity assays.

CD4<sup>+</sup> T lymphocytes. In the vaccinated animals, monkeys H500 and H511 had completely stable CD4<sup>+</sup> T-lymphocyte counts, whereas monkeys T119 and H507 exhibited partial declines in CD4<sup>+</sup> T-lymphocyte counts by day 168 after chal-

lenge. On day 70 after challenge, a time by which the setpoint of viral replication is reached in SHIV-89.6P-infected rhesus monkeys, the CD4<sup>+</sup> T-lymphocyte counts in the vaccinated monkeys were significantly higher than in the control monkeys ( $P = 0.028$  by a two-sided Wilcoxon rank sum test).

We next measured plasma viral RNA levels in the monkeys by a real-time amplification assay with a detection limit of 500 copies/ml (17, 39). As demonstrated in Fig. 5, the control monkeys developed high levels of peak primary viremia, reaching  $5.4 \times 10^7$  to  $3.8 \times 10^8$  copies/ml on day 10 or 14 after challenge. In the vaccinated monkeys, peak primary viremia was slightly lower, between  $4.4 \times 10^6$  and  $1.4 \times 10^8$  copies/ml. On day 70 after challenge, all the control animals had high-setpoint viral RNA levels of  $1.2 \times 10^4$  to  $5.9 \times 10^6$  copies/ml. In three of the four vaccinated monkeys (T119, H500, and H511), setpoint viremia was below the limit of detection of the assay (<500 copies/ml). Setpoint viremia in monkey H507, however, remained high. Interestingly, this animal had the lowest levels of vaccine-elicited CTLs prior to challenge. A trend toward a reduction in setpoint viremia was observed in the vaccinated animals compared with the control monkeys ( $P = 0.11$  by a two-sided Wilcoxon rank sum test). The vaccinated animals had a 2.0-log reduction in geometric mean viral RNA levels after setpoint compared with the control animals.

## A. SHIV-89.6



## B. SHIV-89.6P

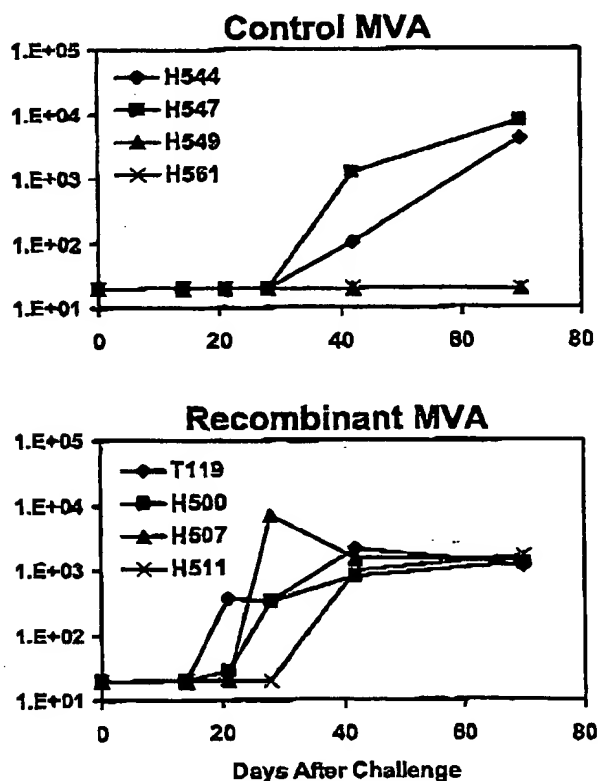


FIG. 3. NAb responses following challenge. Plasma antibody titers capable of neutralizing SHIV-89.6 (A) and SHIV-89.6P (B) were measured by MT-2 cell-killing assays at multiple time points (9).



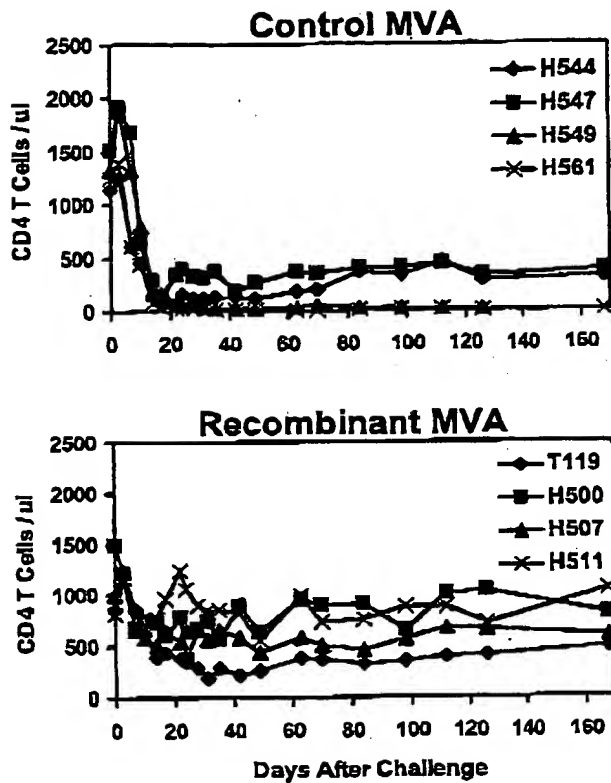


FIG. 4. CD4<sup>+</sup> T-lymphocyte counts following challenge. CD4<sup>+</sup> T-lymphocyte counts in peripheral blood were determined by multiplying the total lymphocyte count by the percentage of CD3<sup>+</sup> CD4<sup>+</sup> lymphocytes at multiple time points.

Significant clinical disease progression was observed in the two control animals (H549 and H561) that had complete depletion of their CD4<sup>+</sup> T lymphocytes, persistent high viremia, and no SHIV-89.6P-specific NAb. These two animals died at days 126 and 168 after challenge. In contrast, all the vaccinated animals remained healthy without evidence of clinical disease or mortality by day 168 after challenge. The rapid development of clinical AIDS and mortality in the control animals is comparable with our previous experience with SHIV-89.6P-infected monkeys (7, 34).

**Immune correlates of protection.** A scatter plot of data shown in Fig. 6 demonstrates a significant correlation between prechallenge vaccine-elicited plateau-phase p11C-specific CTL responses determined by tetramer staining and day 70 post-challenge setpoint viral RNA levels ( $P = 0.03$  by a two-sided Spearman rank correlation test). This correlation is analogous to the correlation we observed in our recent study of immune responses and the protective efficacy elicited by DNA vaccination (7).

## DISCUSSION

In this study, virus-specific immune responses were elicited in rhesus monkeys using recombinant MVA vectors expressing

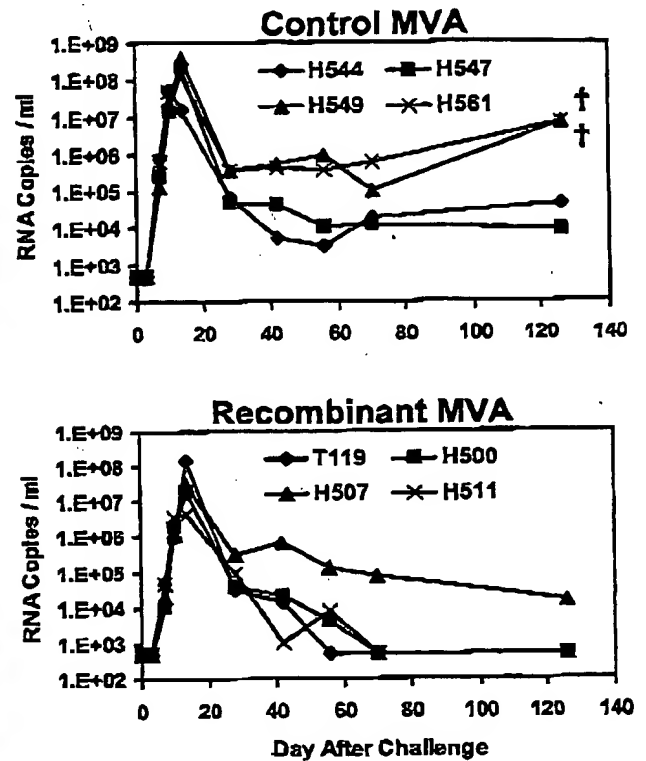


FIG. 5. Viral RNA levels following challenge. Plasma viral RNA levels were determined at multiple time points by a real-time amplification assay with a detection limit of 500 copies/ml (17, 39). †, death of the animal.

SIV *gag-pol* and HIV-1 89.6 *env*. The kinetics and magnitude of the vaccine-elicited SIV Gag epitope-specific CTL responses were comparable to those observed in our previous study of recombinant MVA-vaccinated rhesus monkeys (37, 38). The levels of vaccine-elicited CTL responses in the present study were also comparable to those elicited by plasmid DNA vaccination in our prior studies, but were lower than those elicited by cytokine-augmented DNA vaccination (6, 7, 12).

As we reported previously (7), there was a statistically sig-

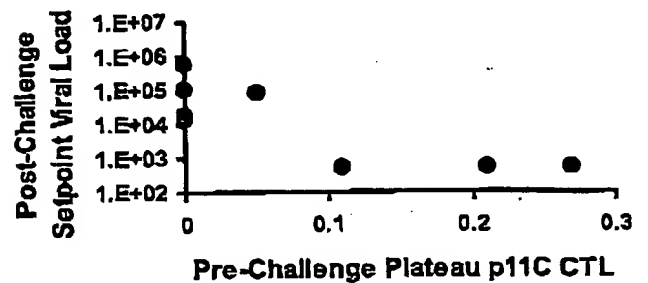


FIG. 6. Correlation of prechallenge vaccine-elicited plateau-phase p11C-specific CTL responses as determined by tetramer staining and day 70 postchallenge setpoint viral RNA levels ( $P = 0.03$ ).

nificant correlation between levels of prechallenge vaccine-elicited plateau-phase CTLs and setpoint viremia following challenge. The asymptotic appearance of the data from the scatter plots in both of these studies suggests that a level of vaccine-elicited plateau-phase CTLs may exist above which little additional benefit is obtained after challenge. The prechallenge plateau-phase CTL population presumably represents the vaccine-elicited memory pool of CTLs which expand upon exposure to virus to become functional effector CTLs. The correlation observed between levels of prechallenge plateau-phase CTLs and the control of viremia following challenge highlights the importance of CTLs in controlling AIDS virus replication.

Following the SHIV-89.6P challenge, secondary SIV Gag epitope-specific CTL responses were clearly evident in the vaccinated animals. The secondary CTL responses were maximal on day 14 and then rapidly declined to steady-state plateau levels. The magnitude of the secondary CTL responses following viral challenge reflected both the levels of vaccine-elicited CTL responses as well as the levels of viral antigen driving these responses. The vaccinated monkey that was unable to control viremia (H507) had persistently high levels of tetramer-binding CD8<sup>+</sup> T cells following challenge, likely reflecting the high levels of antigen present in this animal.

NAbs specific for SHIV-89.6 and SHIV-89.6P were undetectable in the vaccinated monkeys at the time of peak vaccine-elicited immunity or on the day of challenge. However, high-titer SHIV-89.6-specific NAb were detected in the vaccinated animals within 3 weeks after challenge. Since SHIV-89.6-specific NAb are rarely detected in naive animals prior to 6 weeks following infection with SHIV-89.6 or SHIV-89.6P (9, 24), the SHIV-89.6-specific NAb response observed here was most likely an anamnestic response primed by the vaccine. This secondary NAb response following challenge was presumably elicited by either shared epitopes between the Env 89.6 immunogen and the Env 89.6P on the challenge virus or a minor SHIV-89.6 quasispecies present in the SHIV-89.6P challenge stock.

NAb specific for SHIV-89.6P were detected by day 21 to 42 after challenge in the vaccinated monkeys and considerably later or not at all in the control monkeys. It is unclear if the earlier emergence of SHIV-89.6P-specific NAb in the vaccinated animals reflected de novo generation of NAb facilitated by the preserved CD4<sup>+</sup> T-cell help in these animals, affinity maturation of the SHIV-89.6-specific NAb, or a secondary NAb response that was primed by the vaccine. This last possibility is perhaps least likely, since SHIV-89.6-specific NAb have poor neutralizing activity against SHIV-89.6P (9, 25). Regardless of the mechanism, these data demonstrate that the rapid emergence of NAb responses specific for a highly pathogenic primary isolate-like challenge virus did not require immunization with a completely homologous Env construct.

On day 14 after challenge, at the time of peak primary viremia, secondary CTL responses were maximal and SHIV-89.6P-specific NAb were undetectable, suggesting that the initial control of primary viremia in the vaccinated animals was mediated predominantly by CTLs. The subsequent control of viremia, however, likely reflects the effects of both cellular and humoral immune responses. In our prior study utilizing MVA vectors expressing *env* *smH-4* and the related challenge virus

SHIVsmE660, the monkeys vaccinated with MVA/*env* developed secondary SHIVsmH-4-specific NAb after SHIVsmE660 challenge but no convincing secondary SHIVsmE660-specific NAb (28, 29). It is possible that the absence of augmented SHIVsmE660-specific NAb following challenge accounted for the observation that MVA/*gag-pol*, MVA/*env*, and MVA/*gag-pol-env* vaccinations all provided comparable partial control of viremia in that study.

A significant limitation of the present study is the small number of monkeys, which precluded a statistical comparison of clinical disease end points and mortality. However, following the SHIV-89.6P challenge, the control animals developed low-frequency CTL responses, low-titer NAb responses, rapid loss of CD4<sup>+</sup> T lymphocytes, high viral RNA levels, and clinical disease and death in two of the four animals in this group. The monkeys that received the recombinant MVA vaccines developed high-frequency CTL responses, high-titer NAb responses, partial preservation of CD4<sup>+</sup> T lymphocytes, reduced viral RNA levels, and no evidence of clinical disease or mortality by day 168 after challenge. The 2.0-log reduction in mean setpoint plasma viremia observed in this study is similar to the 1.9-log reduction we have reported in SHIV-89.6P-challenged monkeys using plasmid DNA vaccination, although it is less striking than the 3.0-log reduction achieved using cytokine-augmented DNA vaccination (7). The results of the present study are also comparable with the results we obtained with recombinant MVA vaccination in conjunction with an SHIVsmE660 challenge (37, 38).

The degree of protection achieved against SHIV-89.6P-induced AIDS by recombinant MVA vaccination and plasmid DNA vaccination should not be interpreted as evidence that SHIV-89.6P-induced disease is easy to ameliorate. In fact, several other vaccine modalities, including purified recombinant proteins and synthetic peptide vaccines, provide no discernible protection against SHIV-89.6P viremia or clinical disease progression in similarly conducted vaccine trials with rhesus monkeys (N. L. Letvin et al., unpublished data). The fact that SHIV-89.6P infection rapidly leads to immunodeficiency and AIDS in the majority of control monkeys makes this a useful challenge model for assessing the ability of vaccine candidates to provide protection against clinical disease progression in a relatively short time frame.

It is likely that a number of vaccine approaches will ultimately prove to have comparable efficacy in eliciting immune responses, controlling viremia, and preventing clinical sequelae of an AIDS virus infection. Such approaches are likely to include recombinant live vectors (38), plasmid DNA (7), and prime-boost approaches that involve boosting a DNA-primed immune response with a recombinant live vector (2, 15, 19, 35). Many of these vaccine strategies will be tested for their utility in humans over the next several years. If viral replication is similarly reduced in vaccinated humans who are subsequently infected with HIV-1, such individuals may demonstrate slowed disease progression and decreased HIV-1 transmission rates (30). Thus, a vaccine that elicits immunity that is not sterilizing but capable of reducing HIV-1 RNA levels following infection may still provide substantial clinical benefits to human populations.

## ACKNOWLEDGMENTS

We thank Russell Byrum, Bioqual Inc., and Simoy Goldstein for generous assistance.

This study was supported in part by NIH grants AI-85343 and AI-26507.

## REFERENCES

- Allen, T. M., J. Sidney, M.-F. del Guercio, R. L. Glickman, G. L. Linsmeyer, D. A. Wiebe, R. DeMars, C. D. Pauza, R. P. Johnson, A. Sette, and D. I. Watkins. 1998. Characterization of the peptide binding motif of a rhesus MHC class I molecule (Mamu-A\*01) that binds an immunodominant CTL epitope from SIV. *J. Immunol.* 160:6062-6071.
- Allen, T. M., T. U. Vogel, D. H. Fuller, B. R. Motha, S. Steffen, J. E. Boyson, T. Shipley, J. Fuller, T. Hanke, A. Sette, J. D. Altman, B. Moss, A. J. McMichael, and D. I. Watkins. 2000. Induction of AIDS virus-specific CTL activity in fresh, unstimulated peripheral blood lymphocytes from rhesus macaques vaccinated with a DNA prime/modified vaccinia virus Ankara boost regimen. *J. Immunol.* 164:4968-4978.
- Altman, J. D., P. A. H. Moss, P. J. R. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Ball, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94-96.
- Baba, T. W., Y. S. Jeong, D. Pennick, R. Bronson, M. F. Greene, and R. M. Rupprecht. 1995. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* 267:1820-1825.
- Baba, T. W., V. Liska, A. H. Khimani, N. B. Ray, P. J. Dailley, D. Pennick, R. Bronson, M. F. Greene, H. M. McClure, L. N. Martin, and R. M. Rupprecht. 1999. Live attenuated, multiple deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. *Nat. Med.* 5:194-203.
- Barouch, D. H., A. Crish, M. J. Kurda, J. E. Schmitz, X. X. Zheng, S. Santra, J. D. Frost, G. R. Krivulka, M. A. Lifton, C. L. Crabbs, G. Heidecker, H. C. Perry, M.-E. Davies, H. Xia, C. E. Nickerson, T. D. Steinhilber, C. I. Lord, D. C. Montefiori, T. B. Strom, J. W. Shiver, M. G. Lewis, and N. L. Letvin. 2000. Augmentation of immune responses to HIV-1 and simian immunodeficiency virus DNA vaccines by IL-2/gp120 plasmid administration in rhesus monkeys. *Proc. Natl. Acad. Sci. USA* 97:4192-4197.
- Barouch, D. H., S. Santra, J. E. Schmitz, M. J. Kurda, T.-M. Fu, W. Wagner, M. Bilaka, A. Crish, X. X. Zheng, G. R. Krivulka, K. Beaudry, M. A. Lifton, C. E. Nickerson, W. L. Triggiani, R. Punt, D. C. Freed, L. Guan, S. Dube, D. Castmire, A. Simon, M.-E. Davies, M. Chastain, T. B. Strom, R. S. Gelman, D. C. Montefiori, M. G. Lewis, E. A. Emini, J. W. Shiver, and N. L. Letvin. 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytotoxic T-lymphocyte-mediated DNA vaccination. *Science* 290:486-492.
- Belyakov, I. M., L. S. Wyatt, J. D. Ahlers, P. Earl, C. D. Pendleton, B. L. Kelsall, W. Strober, B. Moss, and J. A. Berzofsky. 1998. Induction of a mucosal cytotoxic T-lymphocyte response by intrarectal immunization with a replication-deficient recombinant vaccinia virus expressing human immunodeficiency virus 89.6 envelope protein. *J. Virol.* 72:8264-8272.
- Crawford, J. M., P. L. Earl, B. Moss, K. A. Reimann, M. S. Wyand, K. H. Manson, M. Bilaka, J. T. Zhou, C. D. Pauza, P. W. H. L. Parren, D. B. Burton, J. G. Sodroski, N. L. Letvin, and D. C. Montefiori. 1999. Characterization of primary isolate-like variants of simian-human immunodeficiency virus. *J. Virol.* 73:10199-10207.
- Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers. 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* 258:1938-1941.
- Deacon, N. J., A. Trykin, A. Solomon, K. Smith, M. Lufford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Elliott, C. Chaffield, V. A. Lawson, S. Crowe, A. Maerz, S. Somn, J. Learmont, J. S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, and J. Mills. 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* 270:988-991.
- Egan, M. A., W. A. Charini, M. J. Kurda, G. Voss, J. E. Schmitz, P. Racz, K. Tenner-Racz, K. Manson, M. Wyand, M. A. Lifton, C. E. Nickerson, T. M. Fu, J. W. Shiver, and N. L. Letvin. 2000. Simian immunodeficiency virus (SIV) gag DNA-vaccinated rhesus monkeys develop secondary cytotoxic T-lymphocyte responses and control viral replication after pathogenic SIV infection. *J. Virol.* 74:7485-7495.
- Egan, M. A., M. J. Kurda, G. Voss, J. E. Schmitz, W. A. Charini, C. I. Lord, M. A. Forman, and N. L. Letvin. 1999. Use of major histocompatibility complex class I/polyptide/B2M tetramers to quantitate CD8<sup>+</sup> cytotoxic T lymphocytes specific for dominant and nondominant viral epitopes in simian-human immunodeficiency virus-infected rhesus monkeys. *J. Virol.* 73:5466-5472.
- Greenough, T. C., J. L. Sullivan, and R. C. Desrosiers. 1999. Declining CD4 T-cell counts in a person infected with nef-deleted HIV-1. *New Engl. J. Med.* 340:236-237.
- Hanke, T., R. V. Samuel, T. J. Blanchard, V. C. Neumann, T. M. Allen, J. E. Boyson, S. A. Sharpe, N. Cook, G. L. Smith, D. I. Watkins, M. F. Cranage, and A. J. McMichael. 1999. Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multicpitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. *J. Virol.* 73:7524-7532.
- Hirsch, V. M., T. R. Fuerst, G. Sutter, M. W. Carroll, L. C. Yang, S. Goldstein, M. Platak, W. Elkins, W. G. Alvord, D. C. Montefiori, B. Moss, and J. D. Lifson. 1996. Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *J. Virol.* 70:3741-3752.
- Igarashi, T., Y. Endo, G. England, R. Sadjadpour, T. Matsuo, C. Buckler, A. Buckler-White, R. Pliska, T. Theodore, R. Shihata, and M. Martin. 1999. Emergence of a highly pathogenic simian/human immunodeficiency virus in a rhesus macaque treated with anti-CD8 mAb during a primary infection with a nonpathogenic virus. *Proc. Natl. Acad. Sci. USA* 96:14049-14054.
- Jin, X., D. E. Banner, S. E. Turtletan, S. Lewin, A. Gettle, J. Blanchard, C. E. Perelson, and D. D. Ho. 1999. Dramatic rise in plasma viremia after CD8<sup>+</sup> T cell depletion in simian immunodeficiency virus-infected macaques. *J. Exp. Med.* 189:991-998.
- Kent, S. J., A. Zhao, S. J. Best, J. D. Chandler, D. B. Boyle, and I. A. Ramshaw. 1998. Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant livepox virus. *J. Virol.* 72:10180-10188.
- Knapp, L. A., E. Lehmann, M. S. Pietschmann, J. A. Urvater, and D. I. Watkins. 1997. A high frequency of Mamu-A\*01 in the rhesus macaque detected by polymerase chain reaction with sequence-specific primers and direct sequencing. *Tissue Antigens* 50:657-661.
- Kurda, M. J., J. E. Schmitz, D. H. Barouch, A. Crish, T. M. Allen, A. Sette, D. I. Watkins, M. A. Forman, and N. L. Letvin. 1998. Analysis of Gag-specific cytotoxic T lymphocytes to simian immunodeficiency virus-infected rhesus monkeys by cell staining with a tetrameric major histocompatibility complex class I-peptide complex. *J. Exp. Med.* 187:1373-1381.
- Learmont, J. C., A. P. Geary, J. Mills, L. J. Ashton, C. H. Raynes-Greenow, R. J. Garsia, W. B. Dyer, L. McIntyre, R. B. Oelrichs, R. I. Rhodes, N. J. Deacon, and J. S. Sullivan. 1999. Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. *New Engl. J. Med.* 340:1715-1722.
- Mayer, H., G. Sutter, and A. Mayr. 1991. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *J. Gen. Virol.* 72:1031-1038.
- Müller, M. D., H. Yamamoto, A. B. Hughes, D. I. Watkins, and N. L. Letvin. 1991. Definition of an epitope and MHC class I molecule recognized by Gag-specific cytotoxic T lymphocytes in SIVmac-infected rhesus monkeys. *J. Immunol.* 147:320-329.
- Montefiori, D. C., K. A. Reimann, M. S. Wyand, K. Manson, M. G. Lewis, R. G. Collman, J. G. Sodroski, D. P. Bolognini, and N. L. Letvin. 1998. Neutralizing antibodies in sera from macaques infected with chimeric simian-human immunodeficiency virus containing the envelope glycoproteins of either a laboratory-adapted variant or a primary isolate of human immunodeficiency virus type 1. *J. Virol.* 72:3427-3431.
- Musey, L., J. Hughes, T. Schacker, T. Shea, L. Corry, and M. J. McElrath. 1997. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. *New Engl. J. Med.* 337:1267-1274.
- Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerrudo, A. Hurley, M. Markowitz, D. D. Ho, D. F. Nixon, and A. J. McMichael. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279:2103-2106.
- Ourmanov, I., M. Bilaka, V. M. Hirsch, and D. C. Montefiori. 2000. Recombinant modified vaccinia virus Ankara expressing the surface gp120 of simian immunodeficiency virus (SIV) primes for a rapid neutralizing antibody response to SIV infection in macaques. *J. Virol.* 74:2960-2965.
- Ourmanov, I., C. R. Brown, B. Moss, M. Carroll, L. Wyatt, L. Platner, S. Goldstein, D. Venton, and V. M. Hirsch. 2000. Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV. *J. Virol.* 74:2740-2751.
- Quinn, T. C., M. J. Wawer, N. Sewankambo, D. Serwadda, C. Li, F. Wabwire-Mangen, M. O. Meehan, T. Litalo, and R. H. Gray. 2000. Viral load and heterosexual transmission of human immunodeficiency virus type 1. *New Engl. J. Med.* 342:921-929.
- Redfield, R. R., D. C. Wright, W. D. James, T. S. Jones, C. Brown, and D. S. Burke. 1987. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. *New Engl. J. Med.* 316:673-676.
- Reimann, K. A., J. T. Li, G. Voss, C. Lekuta, K. Tenner-Racz, P. Racz, W. Lin, D. C. Montefiori, D. E. Lee-Parritz, Y. Li, R. G. Collman, J. Sodroski, and N. L. Letvin. 1996. An env gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. *J. Virol.* 70:3198-3206.
- Reimann, K. A., J. T. Li, R. Veazey, M. Halloran, J.-W. Park, G. B. Karlsson,

- J. Sodroski, and N. L. Letvin. 1996. A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate *env* causes an AIDS-like disease after in vivo passage in rhesus monkeys. *J. Virol.* 70:6922-6928.
34. Rehmann, K. A., A. Watson, P. J. Dailey, W. Lin, C. I. Lord, T. D. Steenbekk, R. A. Parker, M. K. Axthelm, and G. B. Karlsson. 1999. Viral burden and disease progression in rhesus monkeys infected with chimeric simian-human immunodeficiency viruses. *Virology* 256:15-21.
  35. Robinson, H. L., D. C. Montefiori, R. P. Johnson, K. H. Manson, M. L. Kallish, J. D. Lifson, T. A. Rivzi, S. Lu, S.-L. Hu, G. P. Mazzara, D. L. Pansicali, J. C. Herndon, R. Glickman, M. A. Candido, S. L. Lydy, M. S. Wyand, and H. M. McClure. 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat. Med.* 5:526-534.
  36. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Saaseville, M. A. Simon, M. A. Lifson, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Chrayeb, M. A. Forman, D. C. Montefiori, E. P. Richer, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8<sup>+</sup> lymphocytes. *Science* 283:857-860.
  37. Seth, A., I. Ourmanov, M. J. Kuroda, J. E. Schmitz, M. W. Carroll, L. S. Wyatt, B. Moss, M. A. Forman, V. M. Hirsch, and N. L. Letvin. 1998. Recombinant modified vaccinia virus Ankara-simian immunodeficiency virus gag pol elicits cytotoxic T lymphocytes in rhesus monkeys detected by a major histocompatibility complex class I/peptide tetramer. *Proc. Natl. Acad. Sci. USA* 95:10112-10116.
  38. Seth, A., I. Ourmanov, J. E. Schmitz, M. J. Kuroda, M. A. Lifson, C. E. Nickerson, L. Wyatt, M. Carroll, B. Moss, D. Venzon, N. L. Letvin, and V. M. Hirsch. 2000. Immunization with a modified vaccinia virus expressing simian immunodeficiency virus (SIV) Gag-Pol primes for an anamnestic Gag-specific cytotoxic T-lymphocyte response and is associated with reduction of viremia after SIV challenge. *J. Virol.* 74:2502-2509.
  39. Suryanarayana, K., T. A. Wiltout, G. M. Vasquez, V. M. Hirsch, and J. D. Lifson. 1998. Plasma SIV RNA viral load determination by real-time quantification of product generation in reverse transcriptase-polymerase chain reaction. *AIDS Res. Hum. Retrovir.* 14:183-189.
  40. Wyand, M. S., K. H. Manson, M. Garcia-Moll, D. Montefiori, and R. C. Desrosiers. 1996. Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. *J. Virol.* 70:3724-3733.
  41. Wyand, M. S., K. H. Manson, M. D. C. Montefiori, J. F. Lifson, R. P. Johnson, and R. C. Desrosiers. 1999. Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. *J. Virol.* 73:8356-8363.
  42. Wyatt, L. S., S. T. Shors, B. R. Murphy, and B. Moss. 1996. Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. *Vaccine* 14:1451-1458.
  43. Wyatt, L. S., S. S. Whitehead, E. A. Venzon, B. R. Murphy, and B. Moss. 1999. Priming and boosting immunity to respiratory syncytial virus by recombinant replication-defective vaccinia virus MVA. *Vaccine* 18:392-397.